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Biochemical investigations into the impact of nitisinone treatment on the metabolome and assessment of whether nitisinone induced hypertyrosinaemia alters neurotransmitter metabolism

Volume I

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"Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem, there is always something you can do and succeed at. It matters that you don't just give up."

Stephen Hawking (1942-2018)

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Abstract

Alkaptonuria (AKU) is a rare inherited inborn error of tyrosine metabolism, resulting from a defect in homogentisate 1,2-dioxygenase. The major metabolic consequence of this is the accumulation of homogentisic acid (HGA) in circulation, and significant excretion in urine. Dating as far back as 1500 B.C. in the Egyptian mummy Harwa, HGA was shown to be central to the pathophysiology of AKU through its deposition in collagenous tissues in a process termed ochronosis. Clinical manifestations are typically observed from the third decade of life and significantly affect the quality of life. Treatment measures available to patients are typically supportive and palliative, and in the last decade a major focus has been on the disease modifying agent nitisinone. This has been shown to be highly efficacious in reducing HGA, and it is hoped this will reduce ochronosis. Its use however has its own impact on metabolism as it leads to marked hypertyrosinaemia, the consequences of which are poorly understood.

Presented herein is a comprehensive evaluation of the impact of nitisinone treatment on the metabolome in AKU. Unique insights into the wider metabolic consequences of treatment are shown through the application of liquid chromatography and mass spectrometry based techniques for the analysis of (i) serum and urine from AKU patients and (ii) urine, plasma, cerebrospinal fluid (CSF) and brain tissue from AKU mice. Novel changes have been demonstrated in relation to tyrosine, tryptophan and purine metabolism, and original observations have been made in relation to the abundance of several metabolites that are associated with oxidative stress.

Importantly the work presented also addresses uncertainty around the impact of hypertyrosinaemia on neurotransmitter metabolism through (i) the quantitative measurement of urinary monoamine neurotransmitter metabolites and their related amino acids in serum from AKU patients, and (ii) the qualitative assessment of monoamine neurotransmitters in CSF and brain tissue from AKU mice. Experiments confirm that hypertyrosinaemia does affect the peripheral metabolism of monoamine metabolites in patients

and mice. Psychometric data collected from AKU patients suggest that hypertyrosinaemia does not lead to depression. Interesting the level of tyramine in brain tissue of AKU mice was shown to increase, whilst dopamine and serotonin levels were unchanged.

The work presented provides a unique example of where studying metabolism can help understand the metabolic consequences of treating a disease. It is hoped that this work will provide a platform for future clinical metabolomic studies into AKU and other complex metabolic diseases.

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List of abbreviations

Below is a list of abbreviations used throughout this thesis. Abbreviations are defined in each Chapter.

AAA	Aromatic amino acid
AADC	Aromatic-L-amino acid decarboxylase
ACN	Acetonitrile
AFAH	Arylformylamine amidohydrolase
Ad	Adrenaline
AKI	Acute kidney injury
AKUSSI	AKU severity score index
AKU	Alkaptonuria
ALDH	Aldehyde dehydrogenase
AM	Accurate mass
AMRT	Accurate mass retention time
BCAA	Branched chain amino acid
BDI-II	Beck's depression inventory-II
CE	Capillary electrophoresis
CEF	Compound exchange file
CI	Confidence interval
CID	Collision induced dissociation
CKD	Chronic kidney disease
CNS	Central nervous system
COAD	Chronic obstructive airways disease
COMT	Catechol-O-methyltransferase
CPMG	Carr-Purcell-Meiboom-Gill
CSF	Cerebrospinal fluid
CT BMD	Computed tomography bone mineral densitometry
CV	Coefficient of variation
DBH	Dopamine-β-hydroxylase
DESI	Desorption electrospray ionisation
DHMA	3,4-Dihydroxymandelaldehyde
DOPAC	Dihydroxyphenylacetic acid
DHPG	3,4-Dihydroxyphenylglycol
ENU	N-ethyl-n-nitrosourea
DPP-TFB	Diphenyl-pyranylium tetrafluoroborate
DP	Dopamine
ESI	Electrospray ionisation
ESRF	End stage renal failure
FAD^+	Flavin adenine dinucleotide
FC	Fold change
FMN	Flavin mononucleotide
FWHM	Fixed width half maximum
G6PD	Glucose-6-phosphate dehydrogenase

GC	Gas chromatography
GP	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GT	γ-Glutamyltranspeptidase
H_2O_2	Hydrogen peroxide
5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxytryptophan
5-HTP	5-Hydroxytryptamine
HDMS	High definition mass spectrometry
HGA	Homogentisic acid
HGD	Homogentisate 1,2-dioxygenase
HILIC	Hydrophobic interaction liquid chromatography
$HO^{\cdot}$	Hydroxyl radical
HPA	4-Hydroxyphenylacetic acid
HPLA	3-(4-Hydroxyphenyl)lactic acid
HPPA	3-(4-Hydroxyphenyl)pyruvic acid
HPPD	4-Hydroxyphenylpyruvate dioxygenase
HRAMS	High resolution accurate mass spectrometry
HT1	Hereditary tyrosinaemia type 1
HVA	Homovanillic acid
I-3-L	Indole-3-lactate
I-3-P	Indole-3-pyruvate
IDO	Indole dioxygenase
IS	Internal standard
KAT	Kynurenine aminotransferase
KH	Kynurenine hydroxylase
KN	Kynureninase
LAT-1	Large neural amino acid transporter-1
LC	Liquid chromatography
QTOF	Quadrupole time of flight
LC-MS/MS	Liquid chromatography tandem mass spectrometry
L-DOPA	L-3,4-Dihydroxyphenylalanine
LNAA	Large neutral amino acid
LLOQ	Lower limit of quantification
LOD	Limit of detection
m/z	Mass to charge ratio
MA	Metadrenaline
MALDI	Matrix assisted laser desorption ionisation
MAO	Monoamine oxidase
MCP	Microchannel plate
3-MT	3-Methoxytyramine
MeOH	Methanol
MetHb	Methaemoglobin
MHPG	3-Methoxy-4-hydroxyphenylglycol
MPP	Mass profiler professional

MS	Mass spectrometry
MSI	Mass spectrometry imaging
MS/MS	Tandem mass spectrometry
NA	Noradrenaline
NAC	National alkaptonuria centre
NACys	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMA	Normetadrenaline
NMDA	N-methyl-d-aspartate
NMR	Nuclear magnetic resonance
NMT	N-methyltransferase
NO	Nitric oxide
NTBC	Nitisinone
NTFA	2-Nitro-4-trifluoromethylbenzoic acid
O_2	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anion
PET-CT	Positron emission tomography-computed tomography
PAH	Phenylalanine hydroxylase
PCA	Principal component analysis
PCDL	Personal compound database and library
PLS	Partial least squares
PLS-DA	Partial least squares-discriminant analysis
PNMT	Phenylethanolamine-n-methyltransferase
ppm	Parts per million
PPP	Pentose phosphate pathway
PQN	Partial quotient normalisation
QA	Quality assessment
QC	Quality control
RBC	Red blood cell
RDA	Recommended daily allowance
RF	Radiofrequency
RNI	Reference nutrient intake
ROS	Reactive oxygen species
RPLC	Reversed phase liquid chromatography
RPo	Resolving power
RT	Retention time
SD	Standard deviation
S:N	Signal to noise ratio
SONIA-1	Suitability of nitisinone in AKU-1
SONIA-2	Suitability of nitisinone in AKU-2
TDO	Tryptophan dioxygenase
TIC	Total ion chromatogram
TEA	Triethylamine
TFA	Trifluoroacetic acid
TOF	Time of flight

TPH	Tryptophan hydroxylase
TyH	Tyrosine hydroxylase
UV	Ultraviolet
VMA	Vallinylmandellic acid
WHO	World health organisation

Part I

Introduction

Chapter 1

General Introduction

Material from published works are included in this chapter:

Davison AS, Hughes AT, Milan AM *et al*., (2020) Alkaptonuria – many questions answered, further challenges beckon. Ann Clin Biochem 57(2):106–120.

Davison AS, Milan AM, Gallagher JA *et al*., (2016) Acute fatal metabolic complications in alkaptonuria. J Inherit Metab Dis 39(2):203-21.

1.1 History of Alkaptonuria

Alkaptonuria (AKU, OMIM 203500) is a rare inherited metabolic disorder of the tyrosine metabolic pathway, which is characterised by a marked increase in circulating and urinary homogentisic acid (HGA). Elevated urinary HGA is diagnostic of AKU and is central to its pathophysiology.

There are several reports of this ancient disease in Egyptian mummies, including Harwa, which is currently housed in the Field Museum in Chicago and dates from 1500 B.C. (Stenn *et al.,* 1977). Scribonius (1584) provided the earliest record of someone with AKU; a school boy that passed urine “as dark as ink”. Twenty-five years later Schenck (1609) reported a similar phenomenon in a Carmelite monk. Similar cases were reported over the next 200 years.

The name alkaptonuria was first introduced by Boedeker who analysed urine from a 44-year old man with lumbar spine pain and poor mobility (Boedeker, 1859). He identified a substance that reduced alkaline copper solutions, but the patient did not have any symptoms of diabetes, and unlike glucose the urinary substance would not reduce alkaline bismuth. He also observed that when left to stand the urine darkened. He further described that this occurred from the surface of the solution downwards and that this darkening accelerated on addition of alkali substances and quickly took up a large volume of oxygen. He described this new substance “alkapton”.

Virchow later provided the first description of pigmented tissues in AKU and coined the term ‘ochronosis’ because of how HGA pigment appeared to be ‘ochre’ under microscopy (Virchow, 1866). Black pigmentation was observed in large weight-bearing joints including intervertebral discs, menisci, laryngeal and tracheal cartilage. The ligaments and synovial tissues displayed pigmentation to a lesser extent than cartilage. When the black pigmentation was observed macroscopically it appeared yellow.

The chemical structure of ‘alkapton’ was identified in 1891 and named HGA (Figure 1.1) because of its structural similarity to gentisic acid (Wolkow *et al.,* 1891).

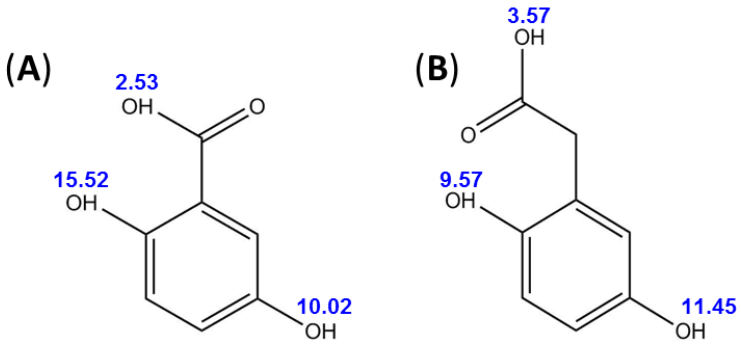

Figure 1.1. Chemical structures and associated pKa’s (denoted in blue) of (A) Gentisic acid and (B) Homogentisic acid. Information on chemical structures from: https://chemicalize.com.

It was in 1902 that Albrecht made the connection between ochronosis of tissues, ochronotic arthritis and AKU. Later in 1904, Osler was the first person to publish evidence to document the diagnosis of AKU in living individuals. The sufferer in question displayed pigmentation of the ears and the sclera, the latter being termed ‘Osler’s sign’.

At this time the disorder was described as the result of an alternative course of metabolism; harmless, usually congenital, and lifelong (Garrod, 2002). Most famously Garrod presented his work at the Croonian lectures in 1908, whereby he introduced the concept of ‘inborn errors of metabolism’ to demonstrate that albinism, AKU, cystinuria and pentosuria were all resultant of Mendelian inheritance, causing variation in metabolism of normal metabolites within normal pathways (Scriver, 2008). Fifty years later, homogentisate 1,2-dioxygenase (HGD, E.C.1.13.11.5), an enzyme involved in the metabolism of tyrosine was identified as the enzymatic defect responsible for AKU (Figure 1.2) (La Du, 1958).

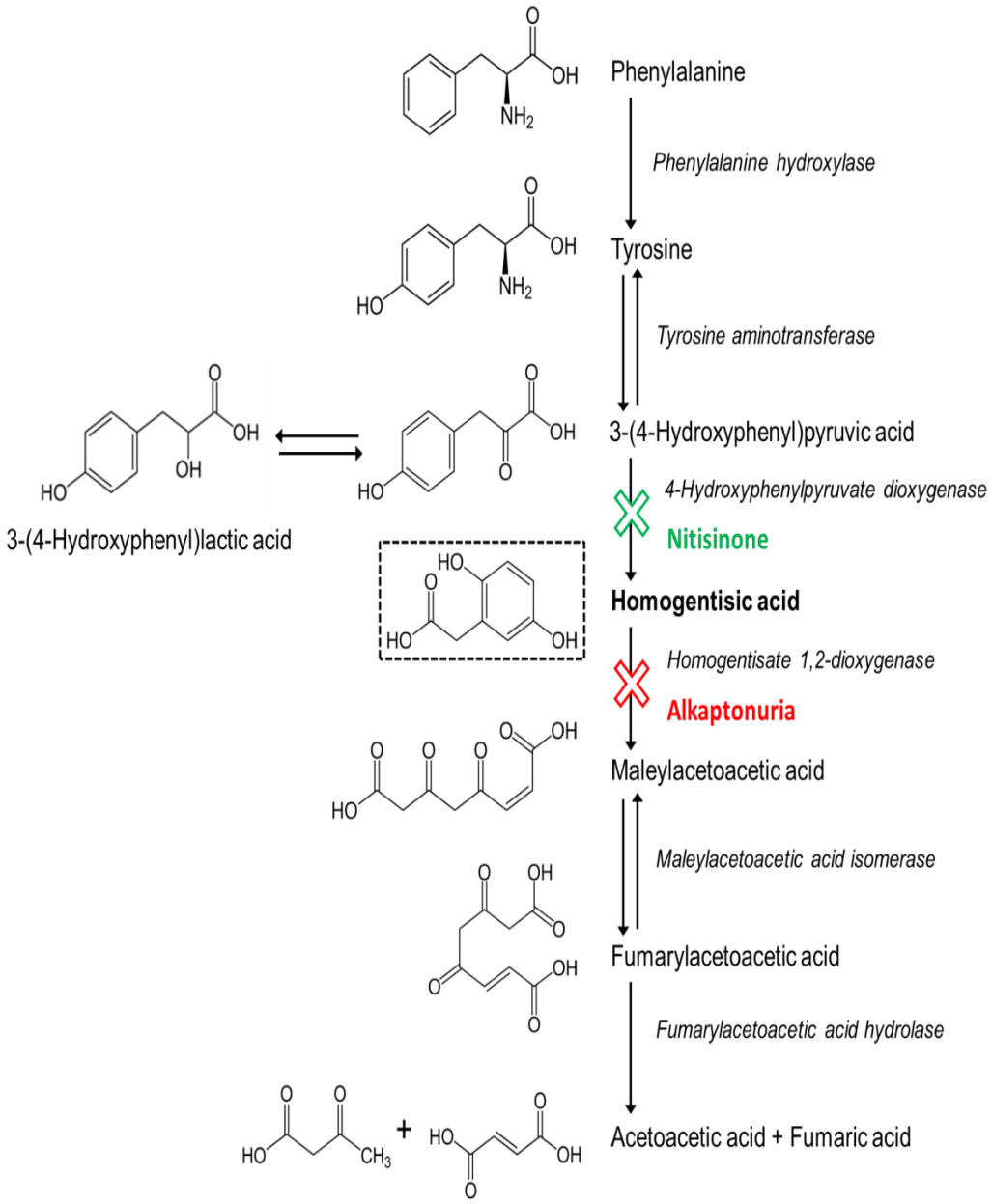

Figure 1.2. Tyrosine metabolic pathway – highlighting the site of the enzyme defect observed in AKU. Adapted from Taylor *et al.* (2018).

1.2 Genetic basis of AKU

The *HGD* gene was first cloned and sequenced from *Aspergillus nidulans* (Fernández-Cañón *et al.,* 1996). The gene maps to the human chromosome 3q13.33 (Gene ID: 3081, http://www.ncbi.nlm.nih.gov/gene) and is a single-copy gene spanning 54 363 bp of genomic sequence, with 14 exons coding

for a protein of 445 amino acids that assembles into a functional hexamer arranged as a dimer of trimers (Titus *et al.,* 2000). Hepatocytes and renal proximal tubular cells are the major sites of HGD activity. AKU arises from homozygous or compound heterozygous mutations in the *HGD* gene. There have been 212 unique AKU mutations identified (*HGD* mutation database: http://hgddatabase.cvtisr.sk, accessed 07/01/2020) of which the most frequent are missense variants (67 %), followed by splicing (12.2 %) and frameshift (12.2 %).

AKU mutations are distributed throughout the entire *HGD* gene with some prevalence in exons 3, 6, 7, 8 and 13 (Zatkova, 2011). Haplotype analysis has been used to trace the migration of specific AKU alleles through human history. The 3 most widespread AKU mutations in Europe, M368V, V300G, and P230S (20, 5, and 5 % of European AKU mutations, respectively) appear to be ancient mutations that were introduced into Europe with the founder populations (Zatkova, 2011). Some *HGD* mutations are found with widespread distribution, examples include S59fs, which was one of the first identified AKU mutations, V300G, as well as the most frequent European mutation M368V (Zatkova 2011). On the other hand, there are mutations rather unique to specific regions; for example c.87+1G>A (p.(Tyr6_Gln29del)) for the Narikuravar gypsy community in India (Sakthivel *et al.,* 2014), A122V in Jordan (Nemethova *et al.,* 2016) and C120W for the Dominican Republic (Goicoechea De Jorge *et al.,* 2002). The largest number of AKU cases (>200) have been reported in Slovakia (Srsen *et al.,* 2002), the majority clustering in a small region in the north west of the country. To date, there is no explanation for this massive increase in the incidence of AKU causing mutations in this region; it cannot be explained by a classical founder effect. In contrast, the high incidence of AKU in the Dominican Republic arose through a classical founder effect with C120W as the mutation (Goicoechea De Jorge *et al.,* 2002).

As yet, no relationship between specific mutations and clinical manifestations has been established (Ascher *et al.,* 2019), although several AKU mutations have been identified, which have been shown to have residual catalytic

activity in functional assays. Interestingly, for 8 AKU patients no *HGD* mutations were identified, and in 22 cases only 1 mutant allele was found (Nemethova *et al.,* 2016).

Recently a large genomic deletion of exon 2 including intronic sequences was reported in 1 case from Lebanon (Zouheir Habbal *et al.,* 2014) and has also been identified in 2 siblings from Israel (Nemethova *et al.,* 2016). It is possible that, in addition to deep intronic mutations, large deletions encompassing 1 or more exons might be occurring in cases where genomic sequencing does not lead to mutation identification.

1.3 Animal models

In 1994, an AKU mouse model generated by n-ethyl-n-nitrosourea (ENU) mutagenesis was identified by Montagutelli *et al.* (1994) due to the presence of darkened cage bedding caused by elevated HGA in the urine. The AKU mutation was backcrossed onto the BALB/cByJ and C57BL/6J murine backgrounds. The murine *HGD* gene was cloned and the mutation identified to be a splice mutation that results in a truncated HGD protein (Manning *et al.,* 1999). This mouse model harbours a recessive splice site mutation, c.1006+2T>A, in exon 10 of the *HGD* gene (GenBank NM_013547) located on mouse chromosome 16. This mutation leads to skipping of 1 or 2 exons and the generation of a premature stop codon. Initially it was reported that the AKU mouse model had the metabolic defects of AKU, but that the tissues did not demonstrate signs of ochronosis. Several theories were suggested including the short life span of the mouse (~2 years) and the endogenous production of ascorbic acid. However, it was subsequently demonstrated that this AKU model (BALB/c $HGD^{-/-}$) had relatively stable elevated plasma HGA concentrations and extensive chondrocyte pigmentation, via a modified Schmorl's stain (Taylor *et al.,* 2012). This model showed that initial pigmentation early in life is pericellular, progressing linearly with age to the intracellular compartment. Treatment with a drug called nitisinone (Figure 1.2) from birth, which inhibits hydroxyphenylpyruvate dioxygenase (HPPD,

EC 1.13.11.27) forming HGA (Figure 1.2), was shown to completely prevent chondrocyte pigmentation in mice (Preston *et al.,* 2014; Keenan *et al.,* 2015).

This *HGD*$^{-/-}$ AKU mouse model has greatly contributed to our knowledge of AKU, in terms of both the disease pathology and treatment, however ENU mutagenesis is not a targeted approach to create a model of genetic disease. Due to the high frequency of DNA mutations that ENU causes there could potentially be other unknown and uncharacterised mutations in this mutagenesis model affecting the disease phenotype. To overcome this uncertainty, a new targeted mouse model of AKU has been raised in the C57BL/6 background using a mutant knockout-first allele obtained from the KOMP repository (www.komp.org). In addition to being targeted, the knockout-first model harbours a lacZ/lacZ transgene for localising gene expression and can be manipulated via FLP/FRT and Cre/LoxP recombination to obtain an inducible and tissue specific knockout. The conditional allele can be used to investigate the effect of partial *HGD* knockout on the AKU phenotype. With gene therapy becoming a likely tool in the future, this conditional mouse model provides an insight into the level and location of *HGD* expression that could rescue the AKU phenotype in humans.

1.4 Clinical manifestations observed in patients

Helliwell *et al.* (2008) demonstrated at autopsy the plethora of features observed in AKU confirming the true multisystem nature of the disease (Figure 1.3). There are few signs and symptoms in the young (Srsen *et al.,* 1982) with symptoms appearing around age 30 years and progressively increasing in severity with age, apart from dark urine which is present from birth (Cox *et al.,* 2011; Ranganath *et al.,* 2011; Ranganath *et al.,* 2013). The various clinical features in AKU can be appreciated from the Alkaptonuria Severity Score Index (AKUSSI) (Table 1.1). This incorporates multiple, clinically meaningful outcomes that can be described in a single score, including kidney and prostate stones, aortic stenosis, bone fractures,

tendon/ligament/muscle ruptures, kyphosis, scoliosis, joint replacements and all other clinical features of AKU.

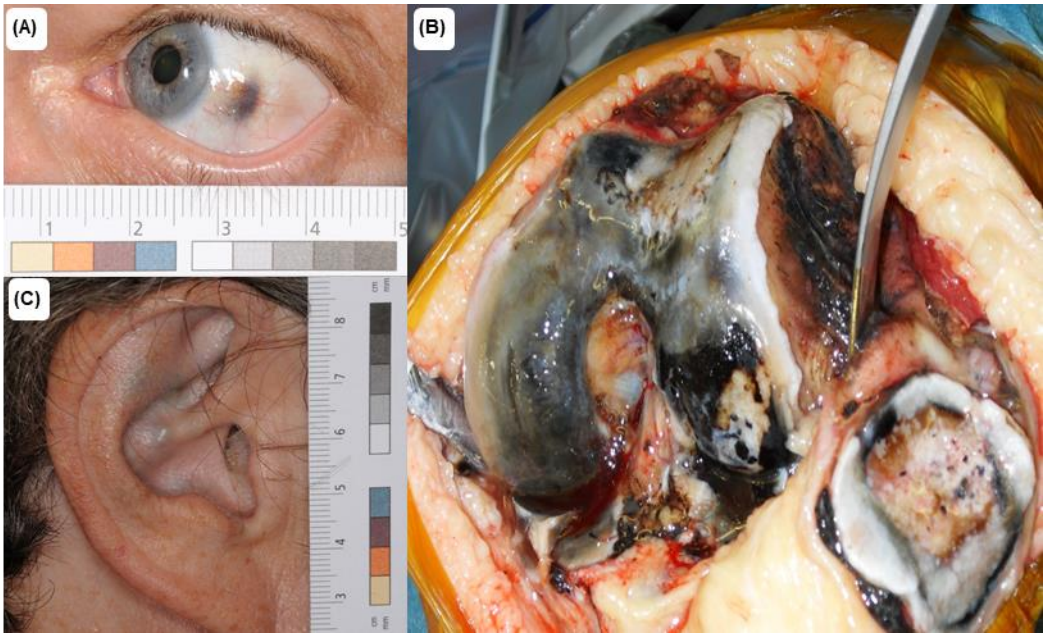

Figure 1.3. Clinical features frequently observed in AKU that should alert a clinician to its possible diagnosis. (A) Pigmentation of the sclera of the eye; (B) Extensive osteoarthritis of the large joints, this is an image of a knee joint at surgery showing pigmentation and fragmentation of the articular cartilage and (C) Slate grey pigmentation of cartilage in the ear (Davison *et al.,* 2020).

Feature	Test	Feature	Test
CLINICAL AKUSSI			
Eye ochronosis: Right eye nasal* Right eye temporal*	Photograph Photograph	**Eye ochronosis:** Left eye nasal* Left eye temporal*	Photograph Photograph
Ear ochronosis: Right ear**	Photograph	**Ear ochronosis:** Left ear**	Photograph
Prostate stones: (4 per episode)	Ultrasound/ History	**Kidney stones:** (4 per episode)	Ultrasound/ History
Osteopenia: (4)	CT-BMD	**Hearing impairment:** (4)	History
Aortic sclerosis: (6), **aortic stenosis:** (mild, moderate, severe) (8, 10, 12)			Echocardiography
JOINT AKUSSI			
Fracture: (8 per fracture)	History	**Muscle rupture:** (8 per rupture)	History
Ligament rupture: (8 per rupture)	History	**Tendon rupture:** (8 per rupture)	History
Joint pain score: (1 for each large joint area; 14 large joint areas)			History
Scintigraphic scan joint score: (2 for each large joint; 14 large joint areas)			^{18F}PET-CT
Number of arthroscopies: (2 each)			History
Number of joint replacements: (4 each)			History
SPINE AKUSSI			
Spinal pain score: (2 each for cervical, thoracic, lumbar, sacroiliac)			History
Scintigraphic scan spine score: (6 areas; 4 points for each area; pubic symphysis, costochondral, lumbar, thoracic, cervical, sacroiliac)			^{18F}PET-CT
Kyphosis: (4)			X-ray
Scoliosis: (4)			X-ray
ALL AKUSSI (CLINICAL + JOINT + SPINE)			

Table 1.1. Summary of Alkaptonuria Severity Score Index. The various clinical features observed in AKU are scored in the manner indicated above. Assessments include subjective pain scoring, photographs, history,

ultrasound abdomen, echocardiogram, computed tomography bone mineral densitometry (CT-BMD), x-ray spine and positron emission tomography–computed tomography (^{18F}PET-CT) scan. *Eye pigmentation: 1, 2 and 3 points for slight, moderate and marked conjunctival pigmentation and 4, 6 and 8 points for scleral pigmentation; **Ear pigmentation: 2 and 4 points for slight and marked pigmentation (Cox *et al.,* 2011; Ranganath *et al.,* 2011; Ranganath *et al.,* 2013).

1.5 Biochemical consequences

AKU is a biochemical defect that arises in the tyrosine degradation pathway (Figure 1.2), whereby deficiency of HGD results in significantly elevated serum and urine HGA (La Du, 1958; Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Sloboda *et al.,* 2019). With advancements in analytical methodologies HGA has been quantified to be present in millimolar concentrations in urine from AKU patients (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Sloboda *et al.,* 2019), this is in contrast to healthy individuals where urinary HGA has been reported as <2.92 *μ*mol/day (Davison *et al.,* 2015). Circulating concentrations of HGA have been shown to be in micromolar quantities, an order of magnitude lower than excreted concentrations (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017). Of note, although AKU is a defect in the tyrosine degradation pathway, serum and urine tyrosine are within normal reference range prior to administration of nitisinone (Phornphutkul *et al.,* 2002; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison, *et al.,* 2018a).

1.6 Management and treatment of AKU

1.6.1 Current treatments available for AKU patients

There is still no approved disease-modifying therapy in AKU. Gene and enzyme replacement therapies are still some way off and currently treatment remains supportive and palliative (Table 1.2). Low protein diet has been used with little effect (de Haas *et al.,* 1998; Phornphutkul *et al.,* 2002) and ascorbic acid used as an anti-oxidant to prevent the conversion of HGA to ochronotic pigment, has unproven efficacy (Phornphutkul *et al.,* 2002). Lifestyle counselling and physiotherapy can be beneficial to patients, but are underutilised.

Supportive analgesia and palliative arthroplasty are crucial to managing the severe musculoskeletal pain in AKU (Rynes *et al.,* 1975; Phornphutkul *et al.,* 2002; Ranganath *et al.,* 2013; Gil *et al.,* 2016). Local anaesthetic patches such as lidocaine are also beneficial. Intermittent colchicine has also been used to control episodic pain (Rynes *et al.,* 1975). Transcutaneous electrical nerve stimulation and acupuncture are widely used to control pain in patients attending the National AKU Centre (NAC). Neuromuscular blocks and trigger point injections can also provide long-term analgesia in AKU. Joint replacement is inevitable, and highly effective. Spinal decompression surgery is needed when spinal compression complicates AKU (Akeda *et al.,* 2008; Gil *et al.,* 2016; Donaldson *et al.,* 2019). Aortic valve disease is highly prevalent and is almost universal by age 60 years in AKU (Pettit *et al.,* 2011). Aortic valve surgery is better carried out electively before left ventricular decompensation ensues, but is technically challenging in ochronotic aortic tissue and sometimes fatal. Stone disease is also common in AKU due to increased HGA. To minimise the formation of renal stones and prevent renal impairment, it is important to emphasize good hydration.

Treatment modality	Mechanism of action	Nature
Joint replacements	Replacing damaged organ	Palliative
Acupuncture	Mediated by endogenous opioids	Supportive
Oral analgesia	Relief from pain depending on drug	Supportive
Parenteral Analgesia	Mainly opioid effect	Supportive
Physiotherapy	Mechanical stretching and strengthening	Supportive
Transcutaneous electrical nerve stimulation	Activation of endogenous opioids	Supportive
Ascorbic acid	Anti-oxidant preventing pigment formation	Unproven as disease modifying
Chondroitin/Glucosamine	Production of glycosaminoglycans	Unproven as disease modifying
Low protein diet	Reduction in tyrosine intake	Some evidence of disease modification

Table 1.2. Supportive and palliative treatment modalities available for AKU. Adapted from Davison *et al.* (2020).

1.6.2 Disease modifying therapy through inhibition of HPPD

Nitisinone (2-[2-nitro-4-(trifluoromethyl)benzoyl]-1,3-cyclohexanedione, $C_{14}H_{10}F_3NO_5$, MW 329.2, Figure 1.4) is a reversible competitive inhibitor of HPPD (half-life ~52-54 h) in the tyrosine metabolic pathway (Figure 1.2) (Lock *et al.,* 1998). Developed as an herbicide, nitisinone was found to be highly efficacious in hereditary tyrosinemia type 1 (HT1, OMIM 276700), and is now standard first-line therapy (Lindstedt *et al.,* 1992; Lock *et al.,* 1998; McKiernan, 2013). The mode of action of nitisinone led to the recognition that it could be effective in AKU. Decreasing circulating concentrations of HGA, through the inhibition of HPPD should in principle decrease ochronosis, thus

preventing the progression of disease in AKU**.** As nitisinone changes the main metabolic abnormality responsible for AKU, it should prevent or reduce its associated morbidity, if started before the symptomatic phase, and alter progression in those already symptomatic. There are several key studies which have looked at the key metabolites in the tyrosine pathway, both before and after a trial of nitisinone therapy (Table 1.3).

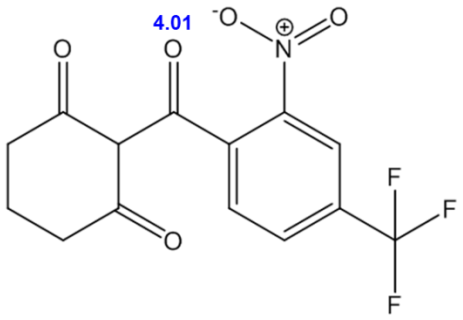

Figure 1.4. Chemical structure and associated pKa (denoted in blue) of nitisinone. Information on chemical structure from: https://chemicalize.com.

Trial design	n	Pre-nitisinone concentrations	Dose	Duration	Overall results	Additional findings	Reference
Open, uncontrolled proof of concept. No dietary modifications	58[#]	$[HGA]_S$ 39.3±15.5 $[HGA]_U$ 2.4-73 $[Tyrosine]_S$ 79±18	0.35-1.4 mg twice daily	9 and 10 days	73-95 % reduction in $[HGA]_U$	Increased urinary HPPA 10-15 fold increase in $[Tyrosine]_S$	Phornphutkul *et al.,* 2002
Open-label, uncontrolled, proof of concept. Reduced protein intake (last week only)	9	$[HGA]_S$ 10.2±6.2 $[HGA]_U$ 23.9±10.6 $[Tyrosine]_S$ 68±18	0.35-1.05 mg twice daily	15 weeks (n=5)	94 % reduction in $[HGA]_U$ Serum HGA below LLOQ	$[HGA]_U$ reduced to ~97 % when protein restricted (<40 g/day) 10 fold increase in $[Tyrosine]_S$	Suwannarat *et al.,* 2005
Randomised, parallel group, single blind	20	$[HGA]_S$ 18.7-62.4 (mean 34.1) $[HGA]_U$ 33.8±11.8 $[Tyrosine]_S$ 60±13	No treatment, 2 mg daily	36 months	>95 % reduction in $[HGA]_U$ 95 % reduction in $[HGA]_S$		Introne *et al.,* 2011
Randomised, open label, parallel-group design	40	$[HGA]_U$ 14.4-69.5	No treatment, 1, 2, 4, 8 mg once daily	4 weeks	98.8 % reduction in $[HGA]_U$ (on 8 mg dose)	10-12 fold increase in $[Tyrosine]_S$ from baseline	Ranganath *et al.,* 2016

Off licence use of nitisinone	28	$[HGA]_S$ 30.0±12.9 $[HGA]_U$ 20.6±5.5 $[Tyrosine]_S$ 42±13.3	2 mg daily after 3 months (2 mg given every other day up to 3 months)	24 months	88.8 and 94.1 % reduction in $[HGA]_U$ at 3 months and 2 years. 83.2 % reduction in $[HGA]_S$ at 2 years	Serum nitisinone 1.58±0.52 μmol/L at 2 years	Milan *et al.,* 2017
Off licence use of nitisinone	3	Maximum $[HGA]_U$ 1211-27 624**	0.2 mg daily. Over duration of study this varied as 1 patient was pregnant and 1 patient was a child	36-60 months	Minimum $[HGA]_U$ 3-882, >90 % reduction	$[Tyrosine]_S$ 305-464	Sloboda *et al.,* 2019

Table 1.3. Summary of clinical studies that have evaluated nitisinone for the treatment of AKU. # - only 2/58 received nitisinone; LLOQ – lower limit of quantification; HPPA – 3-(4-hydroxyphenyl)pyruvic acid. $[HGA]_S$ – serum HGA concentration; $[HGA]_U$ – urinary HGA concentration; $[Tyrosine]_S$ – serum tyrosine concentration; $[Tyrosine]_U$ – urinary tyrosine concentration. All serum and urine HGA and tyrosine concentrations, except where ** displayed, are expressed in μmol/L and mmol/24 h, respectively and as mean ± SD where data were available, otherwise a range is quoted. ** – $[HGA]_U$ expressed as mmol/mol creatinine.

1.6.3 Clinical trials of nitisinone in the USA

In the earliest study of AKU (Phornphutkul *et al.,* 2002) 2 women were given nitisinone at a 30-fold lower dose than that used in HT1 (McKiernan, 2013). Urine and plasma HGA, and serum tyrosine concentrations were measured in all patients pre- and post-treatment. Within this study, there were also 10 patients on ascorbic acid with doses ranging from 0.25-4 g/day (recommended daily dose is 0.065-0.09 g/day, with an upper limit of 2 g). Notable in this study was that there was no correlation between urine HGA excretion and the genetic mutation (Phornphutkul *et al.,* 2002). High dose ascorbic acid treatment has not been proven efficacious (Sealock *et al.,* 1940; Wolff *et al.,* 1989; Phornphutkul *et al.,* 2002). The mean urinary HGA in those on high dose ascorbic acid was not significantly different from the untreated AKU patients. In addition, those on a low protein diet also showed no difference in urinary HGA excretion.

Another open-label study at the National Institute for Health (USA) which, employed nitisinone (2.1 mg daily), demonstrated a 95 % decrease in urinary HGA excretion in 9 AKU adult patients over a 4 month period (Suwannarat *et al.,* 2005). This study also confirmed that a strict protein restriction (1 week only) produced an additional decrease in urine HGA concentrations (Table 1.3). Various definitions of protein restriction led to some of the ambiguity in effects with the recommended daily allowance (RDA) defined as 0.8 g protein/kg of body weight; approximately 56 or 46 g/day for the average sedentary male or female, respectively. Where a reduction in urinary HGA has been quantitated, protein has been restricted to <40 g/day and as low as 20 g/day (Suwannarat *et al.,* 2005), which is not sustainable long-term and would lead to detrimental effects on muscle strength, a side-effect which would worsen these patients' symptoms.

In a third study, a 3-year single-blind clinical trial of 20 patients, the nitisinone group (2 mg/day) showed a sustained decrease in mean urinary HGA from 30.3 to 0.74 mmol/day (Introne *et al.,* 2011). Mean plasma HGA concentrations fell from 34.1 to 1.82 μmol/L after treatment. Urine and plasma HGA decreased by 98 and 95 %, respectively, but despite this the

study was reported as inconclusive as the clinical primary and secondary outcome measures failed to show benefit from the treatment.

1.6.4 Clinical trials of nitisinone in the UK and Europe

The impetus for continued clinical development of nitisinone use in AKU came from Liverpool in the UK. A 2-pronged strategy was adopted: (1) licencing of nitisinone for AKU through clinical trials and (2) use of nitisinone off-label to determine its efficacy and safety in AKU. The first clinical trial was a dose-ranging study (part of a programme called DevelopAKUre, funded by the European Commission as part of the Framework Programme 7) called SONIA-1 (Suitability Of Nitisinone In Alkaptonuria-1) and concluded that nitisinone 8 mg was the dose that decreased urine HGA most efficaciously (Ranganath *et al.,* 2016). Nitisinone (10 mg capsule once daily) was then trialled in SONIA-2 (Suitability Of Nitisinone In Alkaptonuria-2, ClinicalTrials.gov Identifier: NCT01916382); this 4-year outcome study was completed in 2019 where the clinical phase data has led to an application being prepared for submission to the European Medicines Agency in February 2020 to approve the use of nitisinone in AKU.

1.6.5 Off-label use of nitisinone

In 2012, NHS England Highly Specialised Services designated Liverpool as the NAC, and approved the use of a 2 mg daily dose of nitisinone. This off-label use of nitisinone in AKU is supported by a multidisciplinary team of healthcare professionals to ensure the efficacy and safety of nitisinone is monitored. The NAC service is ‘protocolised’ to allow collection of high-quality data on the use of nitisinone as required by NHS England. The 3-year data on the use of nitisinone in the NAC shows partial reversal of ochronosis and slower progression of the disease (Ranganath *et al.,* 2018; Griffin *et al.,* 2018). This is the first time that the disease process and clinical outcomes in AKU have been beneficially modified by any therapy.

Recently Sloboda *et al.* (2019) reported the off-licence use of nitisinone over 3-5 years in 3 patients aged between 3-28 years old. Interestingly these patients were given a very low dose of nitisinone, 0.2 mg/day in an attempt to

reduce HGA and limit hypertyrosinaemia. Remarkably in these patients urinary HGA was reduced by >90 %, and mean serum tyrosine concentrations ranged from 305-416 *μ*mol/L. While these patients were not part of a clinical trial or indeed attending a specialist centre like the NAC in the UK, they had strict monitoring of their protein intake (0.5 g/kg/day – patient 1; 1.1 g/kg/day – patient 2 (pregnant during period reported) and 1.6-2.5 g/kg/day – patient 3 (child)). Unique to this report is treatment through pregnancy and during childhood.

1.6.6 Nitisinone-induced hypertyrosinaemia

1.6.6.1 Magnitude of hypertyrosinaemia

The universal metabolic complication of nitisinone therapy is hypertyrosinaemia; reported in both AKU (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Sloboda *et al.,* 2019) and HT1 (Lindstedt *et al.,* 1992; van Ginkel *et al*., 2016; Zeybek *et al.,* 2017). The dose of nitisinone used in AKU trials is significantly lower compared with that used in HT1; mg/day compared with 1-2 mg/kg body weight in HT1; however the increase in tyrosine is comparable (Table 1.3).

Introne *et al.* (2011) demonstrated that the average serum tyrosine was approximately 800 *μ*mol/L with the highest measured being 1500 *μ*mol/L on a 2 mg daily dose (no dietary restriction). Ranganath *et al.* (2016) added to this with SONIA-1 trial whereby a short-term study examined the effects of varying nitisinone doses (1-8 mg daily) on the metabolic profile. A dose-dependent decrease in urine HGA excretion was measured with a 98.8 % reduction at 8 mg/day. This dose dependence was not reflected in serum tyrosine concentrations; although a 10-fold increase was observed. This supports the findings of Introne *et al.* (2011), with all patients having serum tyrosine >500 *μ*mol/L and the highest 1117 *μ*mol/L (on 4 mg daily dose). Data from the NAC corroborates findings from previous studies; with the longitudinal monitoring of tyrosine metabolites, whilst treated with a 2 mg daily dose of nitisinone, showing a 94 % reduction in urine HGA maintained at 2 years with a concurrent reduction in serum HGA (83.2 %) (Milan *et al.,*

2017). Mean serum tyrosine at 2 years was 594 μmol/L with a large variation reflecting the dietary protein contribution. In contrast Sloboda *et al.* (2019) demonstrated mean serum tyrosine concentrations between 305-416 μmol/L on a 0.2 mg daily dose of nitisinone with a mean reduction of HGA >90 %. This report demonstrates that tyrosine can be kept under 500 μmol/L, on nitisinone therapy; however this was in a small number of patients, one of which was on protein restriction. Furthermore, urinary HGA levels remained relatively elevated compared to previous studies which, is a big limitation in this study.

1.6.6.2 Hypertyrosinaemia and neurotransmitter metabolism

Tyrosine has several metabolic fates beyond that of being metabolised in the tyrosine metabolic pathway (Figure 1.2) depicted in Figure 1.5.
It is a precursor for the biosynthesis of catecholamine neurotransmitters, thyroid hormones and melanin.

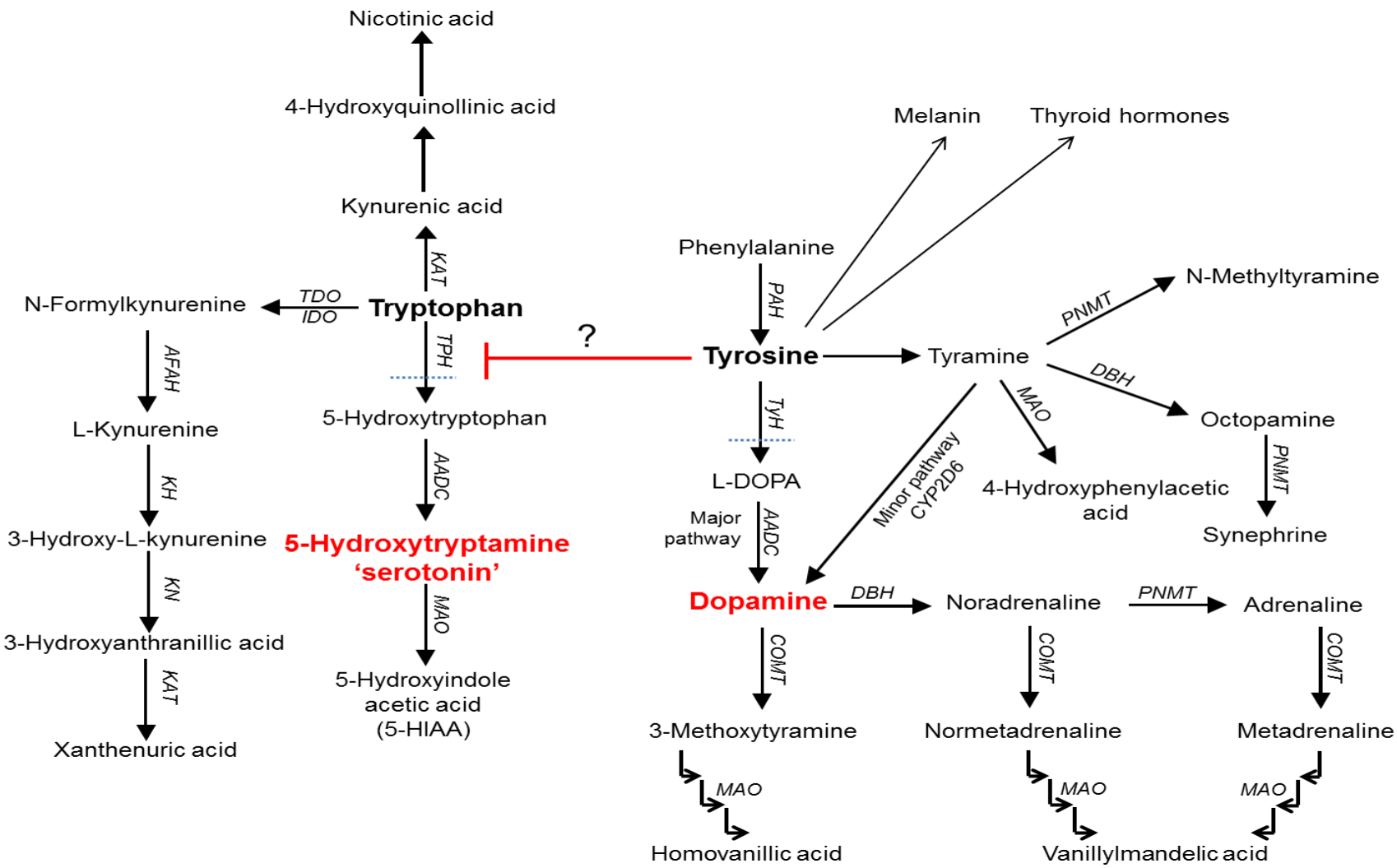
Nicotinic acid
4-Hydroxyquinollinic acid
Kynurenic acid
KAT
N-Formylkynurenine
TDO
IDO
Tryptophan
TPH
?
AFAH
L-Kynurenine
KH
3-Hydroxy-L-kynurenine
KN
3-Hydroxyanthranillic acid
KAT
Xanthenuric acid
5-Hydroxytryptophan
AADC
5-Hydroxytryptamine
'serotonin'
MAO
5-Hydroxyindole
acetic acid
(5-HIAA)
Melanin
Thyroid hormones
Phenylalanine
PAH
Tyrosine
Tyramine
PNMT
N-Methyltyramine
DBH
Octopamine
PNMT
Synephrine
MAO
4-Hydroxyphenylacetic
acid
TyH
L-DOPA
Major
pathway
AADC
Minor pathway
CYP2D6
Dopamine
DBH
Noradrenaline
PNMT
Adrenaline
COMT
COMT
COMT
3-Methoxytyramine
Normetadrenaline
Metadrenaline
MAO
MAO
MAO
Homovanillic acid
Vanillylmandelic acid

Figure 1.5. The metabolic fate of tyrosine in catecholamine biosynthesis and its potential association with tryptophan metabolism in the presence of hypertyrosinaemia. The major neurotransmitters are in red (serotonin and dopamine). PAH – phenylalanine hydroxylase; TyH – tyrosine hydroxylase; COMT – catechol-O-methyltransferase; MAO – monoamine oxidase; PNMT – phenylethanolamine-N-methyltransferase; DBH – dopamine-β-hydroxylase; TPH – tryptophan hydroxylase; AADC – aromatic L-amino acid decarboxylase; AFAH – aryl-formylamine amidohydrolase; ALDH – aldehyde dehydrogenase; KAT – kynurenine aminotransferase; KH – kynurenine hydroxylase; TDO – tryptophan dioxygenase; IDO – indole dioxygenase; KN – kynureninase; NMT – N-methyltransferase; L-DOPA – L-3,4-dihydroxyphenylalanine;--- - indicates rate limiting steps in tyrosine and tryptophan metabolism.

Much of the interest in hypertyrosinaemia comes from studying HT1 and has centred around the direct impact it may have on catecholamine metabolism, specifically dopamine (DP), and the indirect impact it may have on serotonin metabolism (Figure 1.5), respectively. The reason for this interest is that both DP and serotonin play a pivotal role in cognitive function and mood.

In HT1 treatment with nitisinone is commenced from an early age despite the potential risk of cognitive impairment because it has dramatically improved survival rates (Thimm *et al.,* 2012; Pohorecka *et al.,* 2012; Bendadi *et al.,* 2014). Typically serum tyrosine concentrations are regularly monitored and as a guide the aim should be to keep tyrosine concentrations between 200-400 *μ*mol/L up to the age of about 12 years. This is not easy to achieve and some centres allow plasma tyrosine concentrations up to 500 *μ*mol/L (de Laet *et al.,* 2013). There are several mechanisms proposed for the altered cognition observed in HT1, these include: increased transport of tyrosine into the brain; decreased transport of other amino acids into the brain (specifically tryptophan); increased central nervous system (CNS) DP; decreased CNS serotonin, oxidative damage from δ-aminolevulinic acid and succinylacetone or modification of neuronal proteins (Thimm *et al.,* 2011; Hillgartner *et al.,* 2016). It has also been suggested that altered serotonin metabolism may be due to direct inhibition of tryptophan hydroxylase (TPH; EC 1.14.16.4) activity by tyrosine (Figure 1.5) (Thimm *et al.,* 2011; Hillgartner *et al.,* 2016). Barone *et al.* (2019) demonstrated that human tyrosine hydroxylase isoform 1 and human tryptophan hydroxylase 2, expressed in *Escherichia coli* (BL21), exhibited prominent substrate inhibition kinetics and that enzyme activity decreased at elevated tyrosine levels. This supports the earlier hypothesis, but in this study no downstream metabolites were measured (*i.e.* dopamine or serotonin). There is no direct evidence of altered cognition or neurotransmitter metabolism in AKU patients with hypertyrosinaemia.

In a recent study in patients with phenylketonuria (PKU) cerebrospinal fluid (CSF) concentrations of 5-hydroxyindole acetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTP) were shown to be significantly lower compared to non-PKU controls. Moreover, significant negative correlations were observed

between CSF 5-HIAA, homovanillic acid (HVA), 5-HTP and phenylalanine (Pilotto *et al.,* 2019). Lower 5-HIAA and 5-HTP concentrations were also correlated with precuneus and frontal atrophy, and lower HVA concentrations with occipital atrophy, respectively. This study provides further support to the supposition that an excess of large neural aromatic amino acids like phenylalanine can impair the transport of other large aromatic amino acids (*i.e.* tryptophan and tyrosine) into the CNS leading to low neurotransmitter concentrations. This study is unique in that metabolites were measured in CSF, thus providing a true reflection of neurotransmitter concentration in the CNS. For this reason, both CSF and brain tissue are ideal for the assessment of neurotransmitter concentrations (Rajkowska *et al.,* 2013; Paterson *et al.,* 2014). However the collection of CSF via lumbar puncture and brain tissue via biopsy are impractical, and so alternative sample types have been used to assess neurotransmitter concentrations with some degree of success, including plasma (Pan *et al.,* 2018) and urine (Roy *et al*., 1986a; Roy *et al.,* 1986b; Grossman *et al.,* 1999; Hughes *et al.,* 2004). For example, Pan *et al.* (2018) conducted a study into patients with major depressive disorders and assessed a panel of 19 neurotransmitter metabolites in plasma using mass spectrometry based techniques, and showed nine were significantly changed in the major depressive subjects compared to controls. These metabolites were involved in GABAergic, catecholaminergic and serotonergic systems.

As AKU is a rare disease there has been very little reported on the assessment of neurotransmitters or their respective metabolites in the context of depression and altered cognitive function until recently where a short-term study demonstrated increased urinary excretion of the DP metabolite, 3-methoxytyramine (3-MT) and decreased urinary normetadrenaline (NMA) over a 4 week period (Davison *et al.,* 2018b). The increased urinary 3-MT was confirmed in patients attending the NAC over a 2 year period with concentrations increasing 2-fold of the normal reference range (Davison *et al.,* 2018c). The marked increase in 3-MT excretion suggests nitisinone alters peripheral metabolism of catecholamines, more specifically DP. Metabolism of catecholamine neurotransmitters is complex

due to the multiple origins and notably urine concentrations will reflect circulating concentrations, renal uptake and renal synthesis (Eisenhofer *et al.,* 1996). Of interest in this larger cohort there was no concurrent change in Beck's depression inventory-II (BDI-II) scores associated with these patients supporting the renal synthesis hypothesis (Davison *et al.,* 2018c). Fascinatingly, the previous change in NMA was not seen over this longer time period; with the increased patient numbers and monitoring of patients over 2 years the NAC data is likely to reflect changes in those on long term therapy. Reassuringly a study using mass spectrometry imaging demonstrated that monoamine neurotransmitter patterns in brain tissue from a murine model of AKU did not change following short term treatment with nitisinone (Davison *et al.,* 2019a).

1.6.6.3 Reported clinical manifestations of hypertyrosinaemia

Despite careful use of nitisinone, tyrosine ocular keratopathy, as well as skin rash are observed in approximately 5 % of patients treated with the 2 mg dose (Introne *et al.,* 2011; Stewart *et al.,* 2014; Ranganath *et al.,* 2016; Khedr *et al.,* 2018; White *et al.,* 2018). With circulating tyrosine concentrations >800 *μ*mol/L, the solubility of tyrosine is exceeded, and corresponds to ocular tyrosine concentrations of 3500 *μ*mol/L (Lock *et al.,* 2006), the point at which tyrosine crystallises in the cornea leading to corneal keratopathy; similar critical tyrosine thresholds for cutaneous and brain effects have not been described. Published cases have demonstrated that eye symptoms resolve upon cessation of nitisinone and either a lower dose and or protein restriction enables the patient to re-start nitisinone. In HT1, studies have also demonstrated ocular keratopathy (~9 %) in patients on nitisinone treatment (Holme *et al.,* 1998; Gissen *et al.,* 2003).

1.6.7 Rationale for treatment of hypertyrosinaemia

Limiting phenylalanine and tyrosine amino acid intake should mitigate hypertyrosinaemia. The minimum dietary recommendations for optimal health are phenylalanine and tyrosine intakes of 15 mg/kg/day, with an optimal dietary ratio of phenylalanine and tyrosine in mass units of 60:40, similar to the phenylalanine to tyrosine ratio seen in human tissue (Pencharz *et al.,*

2007). The effectiveness of dietary restriction of both tyrosine and phenylalanine has been demonstrated in a murine model of AKU (plasma tyrosine <400 *μ*mol/L after 3 days of restriction) and in patients attending the NAC (4 out of 10 patients had serum tyrosine <700 *μ*mol/L following restriction) (Hughes *et al.,* 2020).

Tackling hypertyrosinaemia by switching to elemental amino acid administration free of phenylalanine and tyrosine is impractical. Instead, the emphasis is on decreasing dietary protein intake to manage hypertyrosinaemia. It is necessary to ensure the reference nutrient intake (RNI) for protein intake is met, the RNI for protein is 0.75 g/kg of body weight in the UK (COMA, 1991) and the RDA for protein is 0.8 g/kg of body weight in the USA (FNB, 2005; WHO, 2007). Meeting these targets for protein should ensure that the requirement for limiting amino acids such as lysine, methionine, threonine and tryptophan are met; such an approach will guarantee adequate provision of other amino acids including phenylalanine and tyrosine.

There is a lack of guidance in the management of hypertyrosinaemia in adults, although there is some guidance in children with HT1 (Pencharz *et al.,* 2007; de Laet *et al.,* 2013). The goal in these children is to maintain serum tyrosine between 200-400 *μ*mol/L and to avoid levels >500 *μ*mol/L (de Laet *et al.,* 2013). Such stringent goals are difficult to achieve in adults with AKU as they have been used to consuming a relatively normal protein intake. At the NAC a pragmatic algorithm-based approach is employed, consistent with existing knowledge and recommendations, where the goal is to maintain circulating tyrosine as low as possible <900 *μ*mol/L. Values of circulating tyrosine of <500 *μ*mol/L are considered desirable and protein intake of 1 g/kg is considered acceptable. Action thresholds for tyrosine have been devised between 500 and 700, and between 700 and 900 *μ*mol/L, invoking lower protein intakes of 0.9 and 0.8 g/kg body weight, respectively (Ranganath *et al.,* 2016; Ranganath *et al.,* 2018), while meeting minimum daily requirements. Concentrations >900 *μ*mol/L require in addition phenylalanine/tyrosine-free meal exchanges.

1.7 Depression and Beck's depression inventory-II

In 2015, the World Health Organization (WHO) reported that 4.4 % of the global population were living with depression (WHO, 2017). Individuals with chronic disease are more likely to experience depressive symptoms (Katon, 2002) and it is well documented in patients with cancer, stroke, heart disease, diabetes, and respiratory disease compared to the general population (Clarke *et al.,* 2009). AKU is a chronic painful debilitating disease making patients more susceptible to depression. Additionally, as alluded to earlier (section 1.6.6.2) there are unsubstantiated concerns about whether nitisinone induced hypertyrosinaemia may lead to altered mood or depression. As a consequence, patients attending the NAC that receive a 2 mg daily dose of nitisinone undergo annual assessments through the Department of Psychology at The University of Liverpool. Assessments include the self-reporting BDI-II questionnaire (Beck *et al.,* 1961; Beck *et al.,* 1996). Whilst this is not a formal assessment of depression like Hamilton Depression Rating Scale or Montgomery-Asberg Depression Rating Scale, it is a widely accepted tool in clinical practice that is cost effective and provides a reasonable quantifiable standard to be compared against (Solomon *et al.,* 2015). It must however be borne in mind that this approach is very subjective in nature as it requires a patient to critically and honestly assess their own behaviour. The BDI-II (Beck *et al.,* 1961; Beck *et al.,* 1996) questionnaire consists of 21 questions where each has 4 alternative statements ranked in order of severity from 0-3. The questionnaire evaluates a number of emotions including: mood, pessimism, sense of failure, self-dissatisfaction, guilt, punishment, self-dislike, self-accusation, suicidal ideas, crying, irritability, social withdrawal, insomnia, and loss of appetite. Conventional total score cut-offs are: 0–13 for minimal depression, 14–19 for mild depression, 20–28 for moderate depression and 29–63 for severe depression, with a maximum score of 63. This approach allows the assessment of the severity and directionality of symptoms, and meets the Diagnostic and Statistical Manual of Mental Disorders DSM-IV criteria (American Psychiatric Association, 1994).

1.8 Acute metabolic complications associated with AKU

Much of the literature on AKU focuses on the chronic metabolic consequences of the disease, and its response to treatment with nitisinone. It is however clear that the metabolic burden of AKU can manifest acutely.

There are 11 cases in the literature reporting the acute fatal metabolic complications that can occur in AKU (Table 1.4). These include 5 patients that had oxidative haemolysis and methaemoglobinaemia (Abero *et al.,* 1983; Suehiro *et al.,* 2007; Isa *et al.,* 2014; Mullan *et al.,* 2015; Freeman *et al.,* 2018); 4 patients with methaemoglobinaemia only (Liu *et al.,* 2001; Uchiyama *et al.,* 2010; Miyasaka *et al.,* 2013; Hugar *et al.,* 2019) and 2 patients with oxidative haemolysis (Heng *et al.,* 2010; Bataille *et al.,* 2014). In all cases the outcome was death.

Common to all of these cases is that all patients had acute kidney injury (AKI), in some cases this was a new presentation and in others it occurred on a background of chronic kidney disease (CKD). Six of 11 patients also presented with sepsis (Liu *et al.,* 2001; Suehiro *et al.,* 2007; Uchiyama *et al.,* 2010; Miyasaka *et al.,* 2013; Mullan *et al.,* 2015; Hugar *et al.,* 2019). As previously mentioned the kidney plays a critical role in the elimination of HGA from the body, and thus it is reasonable to expect significant increases in the circulating concentration of HGA when kidney function is impaired. In all 11 cases reported HGA was not measured, but is presumed to have been increased as kidney function was impaired.

Age (yrs) Gender	Primary Illness	Trigger	MetHb present/max concentration (%)	Treatment	Outcome	Reference
50 Male	Arthropathy Type 2 diabetes CKD	Exacerbation of COAD Metabolic acidosis Haemolysis	Yes 34	Methylene blue	Death	Abero *et al.*, 1983
66 Female	Aortic valve surgery COAD Hereditary telangiectasias Diabetes	AKI Sepsis	Yes No value reported	Unknown	Death	Liu *et al.*, 2001
59 Male	CKD	Exacerbation of CKD Metabolic acidosis Haemolysis Sepsis	Yes 29.9	Haemodialysis Methylene blue Vitamin C Transfusion	Death	Suehiro *et al.*, 2007
79 Male	Arthropathy	AKI Sepsis	Yes 27.9	Transfusion Vitamin C	Death	Uchiyama *et al.*, 2010
24 Male	CKD Hypertension Epilepsy	Exacerbation of CKD Haemolysis Metabolic acidosis	No	Haemodialysis/filtration N-Acetyl cysteine Vitamin C Transfusion	Death	Heng *et al.*, 2010
50 Female	None	AKI Sepsis	Yes 26.8	Haemodialysis Methylene blue Transfusion	Death	Miyasaka *et al.*, 2013

27 Male	CKD Renal transplant Aortic insufficiency ?Erythropoeitic protoporphyria	Haemolysis Thromocytopenia Acute liver failure	No	Haemodialysis Antibiotics Transfusion	Death	Bataille *et al.,* 2014
72 Female	Diabetes mellitus Gastric ulcers Ischaemic heart disease Arthropathy	AKI Dehydration	Yes 43.6	Methylene blue Ascorbic acid Renal replacement therapy Transfusion	Death	Isa *et al.,* 2014
63 Male	CKD Hypertension	Urosepsis Hydronephrosis Calculi Exacerbation of CKD Metabolic acidosis Haemolysis	Yes 25.1	Haemodialysis/filtration N-Acetyl cysteine Vitamin C Transfusion	Death	Mullan *et al.,* 2015
63 Female	1 week history of anorexia, nausea, abdominal pain	AKI Haemolysis Not known to have AKU, diagnosed at post mortem	Yes No value reported	Plasmaphoresis Exchange transfusion	Death	Freeman *et al.,* 2018
60 Female	ESRF Arthropathy Mitral valve replacement Anaemia	3 day history of weakness Anaemia	Yes 24.5	Exchange transfusion Methylene blue Haemodialysis Antibiotics	Death	Hugar *et al.,* 2019

Table 1.4. Cases of the acute haematological complications observed in patients with AKU reported in the literature. CKD – chronic kidney disease; AKI – acute kidney injury; COAD – chronic obstructive airways disease; MetHb – methaemoglobin; ESRF – end stage renal failure. Methaemoglobin reference range <1.5 %.

Introne *et al.* (2002) reported on a patient with AKU and CKD, where an increased plasma concentration of HGA was observed along with worsening clinically apparent ochronosis. Three months post renal transplantation the concentration of HGA was decreased from 126.3 to 43.7 *μ*mol/L demonstrating the importance of kidney function in the elimination of HGA.

Interestingly, no acute oxidative haemolysis or methaemoglobinaemia was reported despite the very high concentrations of plasma HGA. This is in keeping with others where CKD has been reported in AKU and no fatal metabolic complications have been observed (Venkataseshan *et al.,* 1992; Kazancioglu *et al.,* 2004; Faria *et al.,* 2012). This suggests that an elevated concentration of HGA alone is not responsible for the fatal metabolic complications that have been observed in other reported cases (Abero *et al.,* 1983; Liu *et al.,* 2001; Suehiro *et al.,* 2007; Heng *et al.,* 2010; Uchiyama *et al.,* 2010; Miyasaka *et al.,* 2013; Bataille *et al.,* 2014; Isa *et al.,* 2014; Mullan *et al.,* 2015; Freeman *et al.,* 2018; Hugar *et al.,* 2019). It is hypothesised that multiple 'insults' to anti-oxidant defence mechanisms have a cumulative effect and are responsible for the reported haematological complications.

In all published cases where fatal metabolic complications were observed treatments utilised were unsuccessful (Table 1.4). The reasons for this are not entirely clear. Treatments were based around renal replacement therapy (haemodialysis and haemofiltration), blood transfusion, and anti-oxidant therapies including; vitamin C, N-acetyl cysteine (NACys) and methylene blue. Some of these therapies can act as pro-oxidant molecules, thus worsening the metabolic complications.

1.9 Pathophysiology of acute haematological complications observed in AKU

1.9.1 Mechanisms maintaining the pro- and anti-oxidant balance

Oxidative stress is an imbalance between the pro-oxidant reactions and anti-oxidant defence mechanisms, which favours the former. Under healthy physiological conditions, pro-oxidant reactions do not predominate as red blood cells (RBCs) have greater than 250 times more reducing capacity than oxidizing potential (Burak *et al.,* 2008). Some of the consequences of a pro-oxidant environment include damage to DNA, membrane ion transport systems, enzymes, proteins, and lipid peroxidation (Al-Omar *et al.,* 2004; Masella *et al.,* 2005).

Oxidative stress can lead to the generation of free radicals. These are chemical entities that possess an unpaired electron and are formed from a 1 or 2 electron reduction of oxygen. Reactive oxygen species (ROS) include the non-radical hydrogen peroxide and singlet oxygen, as well as the radicals: superoxide anion, hydroxyl radical and nitric oxide (Burak *et al.,* 2008) (Figure 1.6).

Enzymatic systems, principally found within RBCs, act as defence mechanisms against oxidative stress (Figures 1.6 and 1.7). Key enzymes include: glutathione peroxidase, glutathione reductase, catalase, cytochrome b5 reductase, superoxide dismutase, glucose-6-phosphate dehydrogenase (G6PD) and thioredoxin reductase. Non-enzymatic factors that contribute to maintaining the redox state of the body include: (a) vitamins A and E, ubiquinone and melatonin, all of which are lipophilic, and (b) vitamin C, glutathione, uric acid, caeruloplasmin, transferrin and haptoglobin all of which are water soluble.

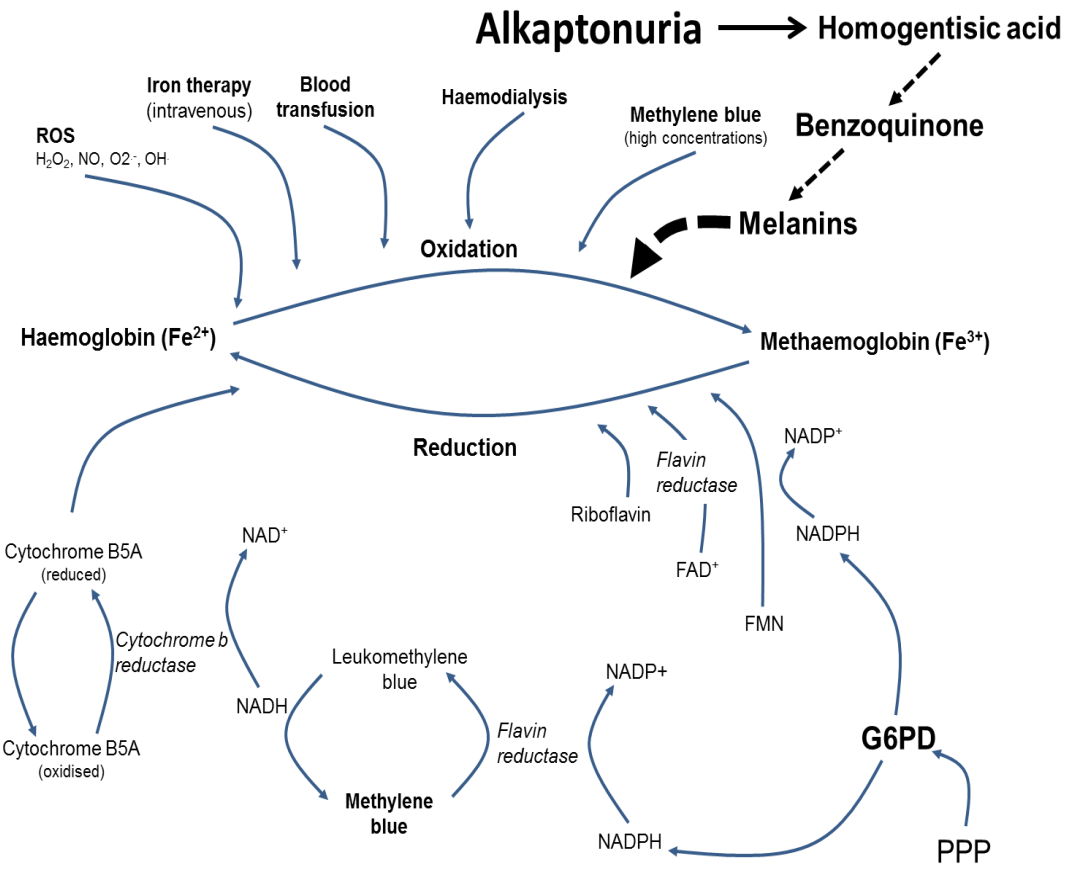

Figure 1.6. Exogenous and endogenous factors influencing the oxidation of haemoglobin. This includes the potential role of HGA and 'soluble melanins' and the biological mechanisms responsible for the reduction of methaemoglobin. ROS – reactive oxygen species; H_2O_2 – hydrogen peroxide; O_2 – singlet oxygen; $O_2^{\cdot -}$ – superoxide anion; $HO^{\cdot}$ – hydroxyl radical; NO – nitric oxide; PPP – pentose phosphate pathway; NADH – nicotinamide adenine dinucleotide; NADPH – nicotinamide adenine dinucleotide phosphate; G6PD – glucose-6-phosphate dehydrogenase; FAD^+ – flavin adenine dinucleotide; FMN – flavin mononucleotide.

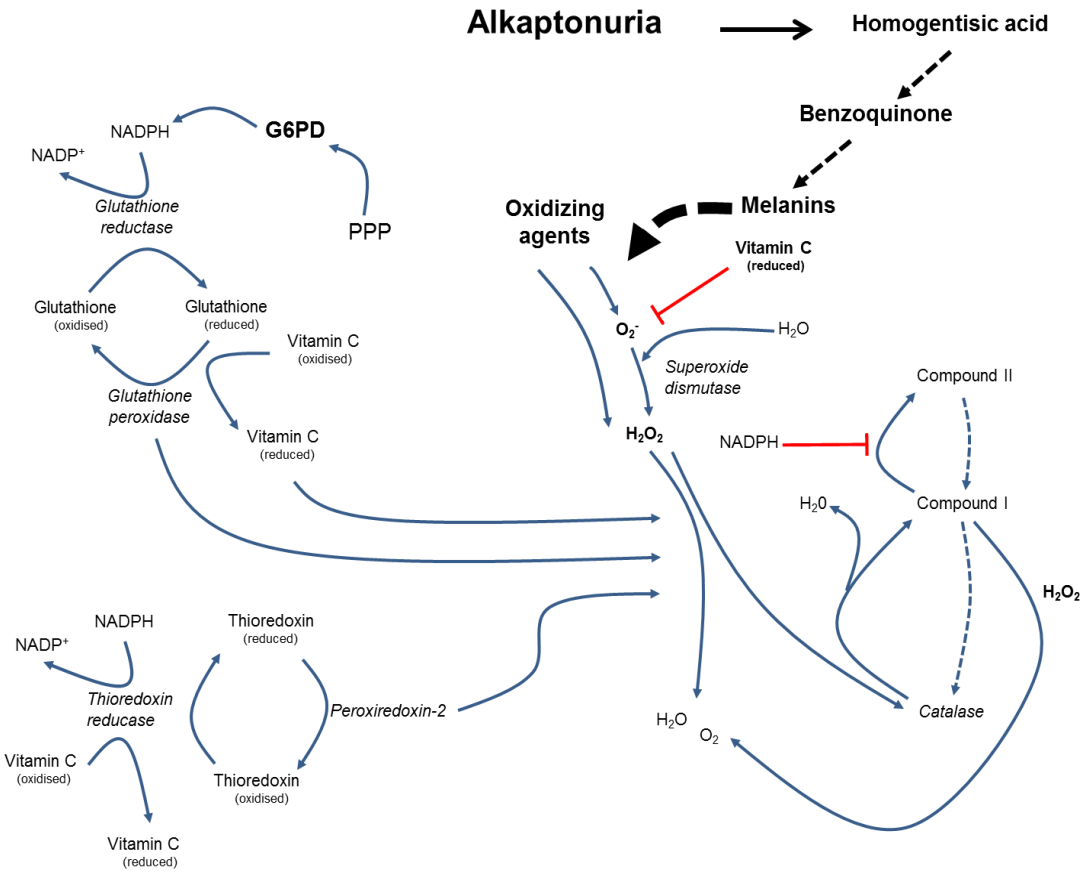

Figure 1.7. Enzymatic systems in the red blood cell responsible for maintaining the redox state of the body. The pathways detailed demonstrate the role of the glutathione, thioredoxin and superoxide dismutase in reducing oxidative species generated in the red blood cell. H_2O_2 – hydrogen peroxide; O_2 – singlet oxygen; $O_2^{\cdot -}$ – superoxide anion; $HO^{\cdot}$ – hydroxyl radical; NO – nitric oxide; PPP– pentose phosphate pathway; NADH – nicotinamide adenine dinucleotide; NADPH – nicotinamide adenine dinucleotide phosphate; G6PD – glucose-6-phosphate dehydrogenase. Red flat ended arrows indicate an inhibitory action.

1.9.2 Disturbance of the pro- and anti-oxidant balance in AKU

Circulating HGA is present at very high concentrations in individuals with AKU (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Sloboda *et al.,* 2019).

HGA undergoes auto-oxidation to form benzoquinone acetic acid polymers, also referred to as 'soluble melanins' (Hegedus *et al.,* 1994). The latter are purported to have pro-oxidant properties. It is proposed that the oxidative stress associated with these melanins and their associated metabolites/free radicals (*i.e.* of oxygen radicals such as superoxide anion, hydroxyl radical, and hydrogen peroxide) are a potential 'trigger' for oxidative haemolysis. Adding vitamin C and glutathione to cultured cells has been shown to reverse oxidative haemolysis (Hegedus *et al.,* 1994).

Increasing evidence to support HGA induced oxidative stress has been reported *in vitro* in serum (Braconi *et al.,* 2011) and cellular models (Braconi *et al.,* 2010), and in patients with AKU (Millucci *et al.,* 2014). Recently a study involving 21 patients with AKU showed that in addition to elevated HGA, urinary total antioxidant capacity and markers of lipid peroxidation (oxidative degradation products of thiobarbituric acid-reactive substances) were significantly elevated compared to controls supporting that increased oxidative stress is observed in AKU. Furthermore, this study reinforced the complexity of oxidative mechanisms occurring in AKU (Albatayneh *et al.,* 2019).

CKD has been shown to reduce the anti-oxidant capacity of the body (Olszewska, 2004; Stepniewska *et al.,* 2005; Stepniewska *et al.,* 2006). However, when kidney function is stable in CKD, acute complications are not frequently observed. Rapid decline of renal function resulting from an acute insult to the body, for example due to an infection or drug toxicity, can lead to AKI (Table 1.4). It is thought that this may predispose an individual to acute oxidative haemolysis.

When AKI occurs in AKU it is proposed that there is a significant retention of HGA, owing to the reduced excretory capacity of the kidney. This in turn may mean that more HGA is oxidised to 'soluble melanins', and an excess of ROS are generated. Together these exceed the anti-oxidant capacity of the body by consuming the substrates required for the anti-oxidant defence mechanisms (*e.g.* NADH, NADPH, vitamin C). This may be exacerbated

further by underlying deficiencies in anti-oxidant defence mechanisms; these may have a genetic (*e.g.* reduced enzyme activity) and or an environmental basis (*e.g.* poor nutrition). Together these factors are likely to contribute to oxidative haemolysis and or methaemoglobin (MetHb) (Figures 1.6 and 1.7), both of which can be fatal as they reduce oxygen carriage to tissues and lead to cyanosis. It is also important to consider that while AKI can occur as a consequence of oxidative stress it can result from massive haemolysis (*e.g.* G6PD deficiency).

Secondary amyloidosis is another important consideration. It is reported by one group to be a complication of AKU and is suggested to contribute to the decline of renal function in AKU (Millucci *et al.,* 2012; Millucci *et al.,* 2014; Millucci *et al.,* 2015). Amyloid A protein is responsible for amyloid A amyloidosis, which occurs in long term inflammatory conditions (Urieli-Shoval *et al.,* 2000). The N-terminal fragment of serum amyloid A is an apolipoprotein synthesised by the liver under the transcriptional regulation of inflammatory cytokines (Simons *et al.,* 2013). In a minority of patients, a sustained inflammatory stimulus and the overproduction of amyloid A protein can lead to protein mis-folding and amyloid deposition (secondary amyloidosis), which may result in kidney damage (Simons *et al.,* 2013; Millucci *et al.,* 2014; Millucci *et al.,* 2015).

In considering the impact of CKD on the pro- and anti-oxidant balance we can get an appreciation of why, when AKI occurs, specifically in patients with AKU the outcome can be fatal. It is clear that there appears to be no single factor that is responsible, rather an intricate interplay of several factors.

In CKD (Olszewska, 2004; Stepniewska *et al.,* 2005; Stepniewska *et al.,* 2006) and AKI (Himmelfarb *et al.,* 2004) the anti-oxidant capacity of the body is reduced. Uraemic toxins, observed in CKD have been shown to be associated with enhanced oxidative stress. This occurs indirectly by carbonyl compounds which are generated from the metabolism of carbohydrates and lipids to form advanced glycation and lipid end products, and by the carbonyl

modification of proteins (Ingani *et al.,* 1999; Miyata *et al.,* 2001). The consequences of this are observed in dialysis related amyloidosis.

Treatment of uraemic patients with haemofiltration or haemodialysis has also been shown to increase oxidative stress and to reduce the concentration of anti-oxidants in the blood (Olszewska, 2004; Stepniewska *et al.,* 2005; Stepniewska *et al.,* 2006). In addition, lack of vitamins A and E, selenium, blood transfusion and parenteral iron administration have been shown to promote a pro-oxidant environment (Agarwal *et al.,* 2004; Olszewska, 2004; Stepniewska *et al.,* 2005; Stepniewska *et al.,* 2006; Karkouti, 2012). More specifically it has been shown that in the RBCs of patients with CKD the pentose phosphate pathway (PPP) does not generate adequate supplies of reductive equivalents (*i.e.* NADPH) necessary to restore reduced glutathione (GSH) (Stepniewska *et al.,* 2006), an important free radical scavenger, thus increasing oxidative stress.

Furthermore, as haemodialysis causes a significant loss of glucose, which is essential for the PPP, significant changes in the anti-oxidant system of the blood of patients with CKD occurs. It has been demonstrated that the activity of RBC glutathione peroxidase decreases with the duration of dialysis, thus potentiating the effects of oxidative stress (Stepniewska *et al.,* 2006). Lower RBC concentrations of selenium in dialysed patients have also been shown to correlate with lower glutathione peroxidase activities (Stepniewska *et al.,* 2006).

RBCs in patients with CKD are also exposed to an increased activity of free radicals. The net consequence of this is that peroxidation of lipids and proteins in RBC membrane occurs (Ceballos-Picot *et al.,* 1996). This reduces the stability of the RBC and increases the likelihood of haemolysis. Advanced oxidation protein and glycation end products also contribute to this and increase the likelihood of haemolysis (Ceballos-Picot *et al.,* 1996).

Disturbances of anti-oxidant enzyme activity in other metabolic pathways can also occur. These include reactions that involve superoxide dismutase, glutathione peroxidase, catalase and glutathione reductase (Figure 1.7). The

extent to which these pathways are altered is determined by the stage of CKD and efficiency of treatment (Stepniewska *et al.,* 2005). Of particular note is that the energy production in RBCs depends largely on glycolysis, with glucose as the principal substrate. Both glycolysis and the PPP generate NADH and NADPH, respectively to reduce MetHb, which is being continuously produced. If aberrations occur in these systems a pro-oxidant environment will dominate and MetHb will be present at higher concentrations (normal reference range <1.5 %, fatal >70 %).

In health there are 2 main mechanisms to minimise the formation of MetHb (Skold *et al.,* 2011). The first is to minimise the formation of ROS and the second to reduce ferric (Fe^{3+}) iron back to its ferrous (Fe^{2+}) state. The main enzyme in RBCs that does this is cytochrome b5 reductase, which utilises NADH to reduce cytochrome b5 which in turn reduces MetHb (Skold *et al.,* 2011).

A second enzyme called flavin reductase also contributes to maintaining haemoglobin in the ferrous state through its utilisation of NADPH and the co-factor methylene blue, to form leukomethylene blue. Riboflavin, flavin adenine dinucleotide and flavin mononucleotide also act as electron acceptors to reduce MetHb back to its ferrous state.

A very important consideration in RBC oxidative stress is the activity of the enzyme G6PD. This enzyme is central to the formation of the reducing equivalents NADH and NADPH. A deficiency in G6PD can lead to inadequate formation of NADPH and NADH, the consequence of which is that the demands of anti-oxidant regulatory pathways are not met. This predisposes an individual to an increased risk of haemolytic anaemia and or methaemoglobinaemia (Cappellini *et al.,* 2008). In all cases reported (Table 1.4) the activity of G6PD was not documented, and may have been an independent risk factor for the development of the fatal metabolic complications observed in AKU.

1.9.3 Treatment options employed for haemolysis in AKU

All therapeutic strategies employed in the 11 cases reported (see Table 1.4) failed to demonstrate therapeutic benefit, as all patients died. It is important to consider why this is the case. Treatments included vitamin C, NACys, methylene blue, blood transfusion and renal replacement therapy (*i.e.* haemodialysis and or haemofiltration). Regardless of treatment it is clear that the supply of reducing equivalents were inadequate to meet the cellular demands to minimise oxidative stress, thus oxidative haemolysis and or the formation of MetHb occurred.

Increased oxidative stress from the generation of 'soluble melanins' (pro-oxidants) may have saturated anti-oxidant mechanisms causing depletion of NADPH and thus when these treatments were given they were ineffective. Both vitamin C and NACys (Figure 1.7) are key co-factors in the recycling of thioredoxin and glutathione, respectively. Vitamin C is a self-limiting anti-oxidant mechanism in humans as they cannot synthesise it endogenously. Moreover, vitamin C when given at high concentrations has been shown to induce haemolysis in patients with G6PD deficiency (Cappellini *et al.,* 2008). No patients were reported to have G6PD deficiency; however this may have been a contributing factor in the lack of therapeutic response to anti-oxidant therapy.

The use of NACys is entrenched in the medical literature for the treatment of oxidative liver damage observed in paracetamol overdose. This is because of its role in the recycling of glutathione. While its use in AKU has not been assessed for clinical efficacy, it has been used based on its purported benefits as an anti-oxidant (Heng *et al.,* 2010; Mullan *et al.,* 2015). It is likely that therapeutic benefit has not been demonstrated in patients with AKU due to the magnitude of pro-oxidant species present.

Methylene blue has a number of clinical uses, but at first glance seems counter intuitive as therapy for the treatment of oxidative stress in AKU as it can behave as a pro-oxidant at higher concentrations, thus potentially exacerbating oxidative stress. In addition, there are concerns over its use in

patients that have G6PD deficiency as it can lead to life threatening haemolytic anaemia (Beutler *et al.,* 1963). It is proposed that leukomethylene blue can diffuse into G6PD deficient RBCs resulting in haemolysis (Beutler *et al.,* 1963).

Haemodialysis and or haemofiltration were used as treatments in 8 of the 11 cases reported (Suehiro *et al.,* 2007; Heng *et al.,* 2010; Miyasaka *et al.,* 2013; Bataille *et al.,* 2014; Isa *et al.,* 2014; Mullan *et al.,* 2015; Freeman *et al.,* 2018; Hugar *et al.,* 2019). While this in principle may have facilitated the removal of HGA, thus potentially reducing the formation of 'soluble melanins' and oxidative stress, it is known that haemodialysis increases oxidative stress as anti-oxidant defences are reduced by pro-oxidant haemodialysis factors, which is more profound in patients with renal anaemia (Olszewska, 2004; Stepniewska *et al.,* 2005; Stepniewska *et al.,* 2006).

As a consequence of haemolysis the majority of patients (Table 1.4) were given blood transfusions. This is a lifesaving treatment during haemolysis; however it is known that transfusions can harm the kidney. The exact mechanism of how this occurs is not fully understood. It is known that RBCs during storage undergo biochemical and morphological change and it is thought that after transfusion these changes promote a pro-inflammatory state impairing oxygen delivery to tissues and promoting oxidative stress (Karkouti *et al.,* 2012).

It is clear that there were limitations to all medical therapies given in the cases reported. In principle renal transplantation could serve to reduce the circulating concentration of HGA (Introne *et al.,* 2002) and thus reduce the risk of oxidative haemolysis and or the formation of MetHb, however this is not a treatment that could be implemented rapidly and has its own challenges (*i.e.* risk of graft rejection). A potentially lifesaving therapy could be the utilisation of a therapy that decreases the production of HGA and this can be achieved by a drug, namely nitisinone.

If the additional ‘insult’ for the haematological complications in AKU is the formation of pro-oxidants as a consequence of HGA accumulating and being metabolised to ‘soluble melanins’ then the inhibition of the formation of HGA through the use of nitisinone would be a highly suitable treatment. Moreover, the drop in HGA concentrations is rapid, with approximately 60 % decrease in circulating HGA within 48 h on 2 mg dose (unpublished data, Ranganath LR) and thus is an efficacious treatment. Published trials have demonstrated a significant dose response decrease in urinary HGA in AKU with 8 mg daily dose reducing urinary HGA to 98.8 % of baseline concentrations (Ranganath *et al.,* 2016, Milan *et al.,* 2019).

It is postulated that treatment with nitisinone should be made available earlier even if renal function is normal rather than waiting for decline in renal function and the presentation of haematological complications. This approach is also recommended as there may be delays in obtaining nitisinone quickly, in what could be a rapidly fatal event.

It is clear from the literature that the metabolic consequences of AKU as a chronic disease entity, and the impact of treatment with nitisinone has largely focused on the targeted measurement of circulating and urinary HGA and tyrosine in a clinical trial setting. Moreover, in reported cases where acute fatal metabolic complications have been observed much of the literature relates to inference based metabolic changes that are observed in other disease states that are known to have increased oxidative stress. It is therefore clear that much is still not know about the wider metabolic consequences of AKU and treatment with nitisinone (*i.e.* the impact of hypertyrosinaemia on neurotransmitter metabolism), and what metabolic changes occur with respect to oxidative stress. It is essential that the wider metabolic consequences of AKU, and nitisinone therapy are investigated further using a different analytical strategy to assess the metabolome.

1.10 Metabolomics

Metabolomics is defined as “global unbiased analysis of the small-molecule metabolites present within a biological system in an identified and quantified manner” (Fiehn, 2002). Small molecule metabolites are typically regarded as having a molecular weight of <1500 Da.

Metabolomics can be split into targeted and untargeted. The targeted approach encompasses the quantitative measurement of a selection of known metabolites from a biochemical pathway, in contrast untargeted metabolomics is defined as the global unbiased analysis of all small molecules that constitute the metabolome (Fiehn, 2002; Patti *et al.,* 2012; Naz *et al.,* 2014; Tzoulaki *et al.,* 2014; Cajka *et al.,* 2016). Non-targeted methodologies can also be subdivided into metabolic profiling, which focuses on specific classes of compounds, and metabolic fingerprinting, which represents a global approach based on the measurement of metabolic patterns (Dettmer *et al.,* 2007).

Metabolomics has seen huge growth in the last decade and its application offers real promise to clinicians as it has the potential to provide predictive, prognostic, diagnostic, and surrogate markers of disease and the potential to inform on the underlying molecular mechanisms of disease (Berger *et al.,* 2016). Ultimately in the clinical environment one of the key aims of metabolomic studies is biomarker discovery, with the hope that new biomarker(s) have the clinical utility to allow specific treatment(s) and or further investigations to be carried out for a patient, providing so called ‘personalised medicine’.

The WHO defined a biomarker as “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction” (WHO, 1993). Numerous factors can influence the metabolome (Figure 1.8,

adapted from Beger *et al.,* 2016) and should be borne in mind when evaluating the utility of a biomarker.

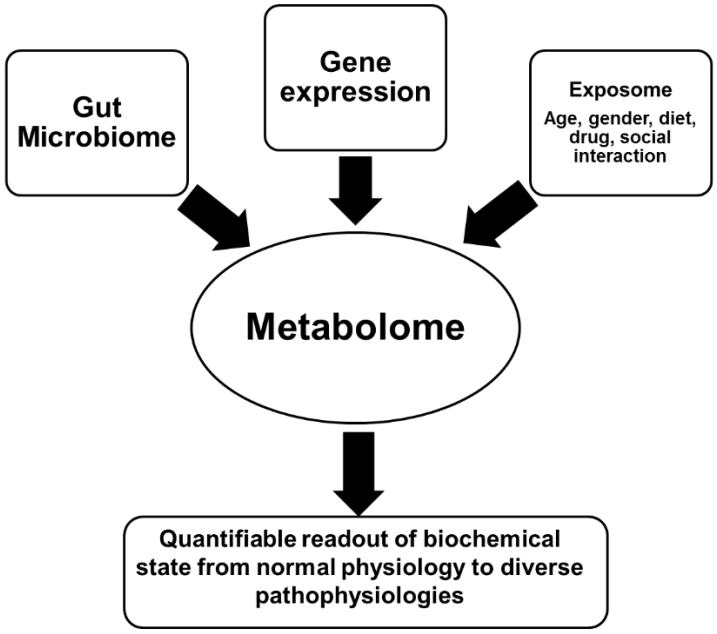

Figure 1.8. Factors affecting the metabolome. Adapted from Beger *et al.* (2016).

1.11 Analytical techniques used in metabolomics

Numerous analytical platforms have been utilised for metabolomic applications. These include nuclear magnetic resonance (NMR), fourier transform infrared spectroscopy and high resolution accurate mass spectrometry (HRAMS) coupled to separation techniques like gas or liquid chromatography, capillary electrophoresis and fourier transform. HRAMS can also be used to evaluate the metabolome of tissue samples, where ions liberated from tissue during the ionisation process (*e.g.* desorption ionisation, electrospray ionisation, DESI) are drawn into the mass spectrometer for analysis. The focus hereafter will be on 1D proton (^{1}H) NMR and HRAMS as these techniques were used in the work presented herein. For a detailed review of other techniques see a recent review by Zhang *et al.* (2012).

There are key advantages and disadvantages to using NMR and HRAMS for metabolomics, these are summarised in Table 1.5 (information taken from Emwas, 2015). In addition to the challenges encountered with the analytical platform chosen, study design (Kirwan *et al.,* 2018), quality control (Broadhurst *et al.,* 2018) and the validation of analytical and chemometric methodologies require careful consideration (Beckonert *et al*., 2007; Beckonert *et al*., 2010; Want *et al*., 2010; Dunn *et al.,* 2011; Want *et al.,* 2013).

	Nuclear magnetic resonance	High resolution accurate mass spectrometry
Advantages	Non-destructive	Widely available technology
	Intrinsically quantitative	High sensitivity (pg-ng)
	Minimal sample preparation	High resolution
	Robust and very reproducible	High metabolite detection (500+)
	Can use for direct analysis of any sample type (including tissue)	Can be combined with a variety of hyphenated techniques (*e.g.* LC/GC/CE)
	Can measure all metabolites under same conditions as long as above NMR sensitivity threshold	Small sample volume required
Limitations	Moderate resolution due to signal overlap	Destructive method
	Low sensitivity (μg)	Moderate reproducibility with salty mixtures due to ion suppression
	Low metabolite detection (40-200)	Detection bias (*i.e.* positive or negative polarity)
	Very expensive	Need different chromatography conditions to detect different metabolite classes
		Sample preparation can be complex

Table 1.5. Comparison between NMR and MS based methodologies for use in metabolomics experiments. GC – gas chromatography; LC – liquid chromatography; CE – capillary electrophoresis. Adapted from Emwas, (2015).

1.11.1 High resolution accurate mass spectrometry

HRAMS provides the ability to screen a sample, whether it be human serum or urine, or even tissue culture media on the basis of exact mass, allowing measurement of the mass to charge ratio (*m/z*) of an ion to the 4th or 5th decimal place. This level accuracy enables isobaric compounds of the same 'nominal', but differing exact masses to be distinguished. However this technique alone cannot distinguish isomers, often this is addressed through the use of hyphenated chromatographic techniques (Wood, 2019).

The International Union of Pure and Applied Chemistry defines resolution in mass spectrometry in 2 different ways (Murray *et al.,* 2013), herein the favoured definition for HRAMS (Barrow *et al.,* 2005) is presented and defines it as m/Δm, where m is the mass of the ion of interest and Δm is the peak width (typically at 50 % of peak height) (Figure 1.9). The resolving power of HRAMS instruments these days is typically ≥10000 m/△m (Table 1.6) (García-Reyes *et al.,* 2017).

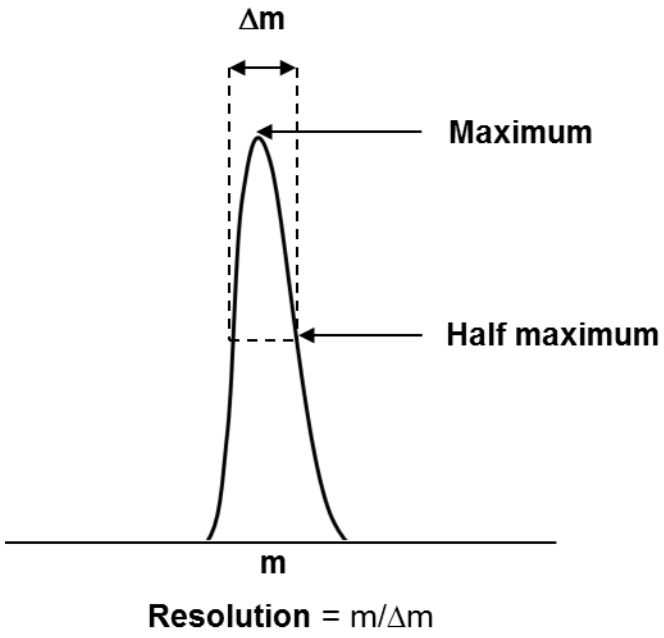

Figure 1.9. Definition of mass resolution. m – mass of the ion of interest; Δm – peak width at 50 % of peak height.

A number of different analytical platforms are available to perform HRAMS, typically time of flight (TOF) and orbitrap analysers are used in metabolomics studies. Table 1.6 summarises technical specifications and characteristics of TOF and orbitrap (García-Reyes *et al.,* 2017) and Table 1.7 their major advantages and disadvantages, respectively (Wood, 2019). From this point forward the focus relating to HRAMS will be on TOF mass spectrometry (TOF-MS) as this was the technique utilised for metabolomic studies presented herein.

Analyser	Manufacturer	Instrument name	Resolving power [FWHM at defined (*m/z*)]	Mass accuracy (ppm)		Mass range (Da)	Acquisition speed (Hz)
				Internal calibration	External calibration		
Q-TOF	Bruker Daltonics	MicroOTOF-QII	20,000 (922)	<2	<5	50-20,000	20
		MaXis impact	40,000 (386)	<1	<3	50-20,000	50
		MaXis 4G	60,000 (1222)	<0.6	<2	50-20,000	30 (MS) 10 (MS/MS)
	Waters	XEVO G2 Q-TOF	22,500 (956)	<1	-	20-16,000	30
		Synapt G2-S HDMS	50,000 (956)	<1	-	20-100,000	30
	Agilent	**6500 Q-TOF**	**42,000 (922)**	**<1**	**-**	**50-10,000**	**50**
	Sciex	TripleTOF 4600	30,000 (full-range)	<0.5	<1	5-40,000	100
		TripleTOF 5600	35,000 (full-range)	<0.5	<2	5-40,000	100
		TripleTOF 6600	40,000 (full-range)	<0.5	<2	5-4000	100
IT-TOF	Shimadzu	LC-MS-IT-TOF	10,000 (1000)	3	5	50-5000	10
Orbitrap	**Thermo Scientific**	Exactive		<1	<5	50-4000	12 at RP 17,500
Q-Orbitrap		**Q-Exactive**	**140,000 (200)**	**<1**	**<5**	**50-4000**	**12 at RP 17,500**
LTQ- Orbitrap		Orbitrap elite	240,000 (400)	<1	<3	50-4000	8 at RP 17,500
Tribrid-Orbitrap		Orbitrap fusion Lumos tribrid	500,000 (200)	<1	<3	50-6000	18 at RP 17,500

Table 1.6. Technical specifications and characteristics of TOF and orbitrap mass analysers. RP – resolving power; FWHM – full width at half maximum; HDMS – high-definition mass spectrometry; IT-TOF – ion trap time-of-flight; LTQ-Orbitrap – linear ion trap orbitrap; MS – mass spectrometry; MS/MS – tandem mass spectrometry. Adapted from Garcia-Reyes *et al.* (2017). **Bold** text indicates mass spectrometers that were used in Chapters 2, 3, 7 and 8.

Analyzer	Advantages	Disadvantages
TOF	Resolving power is independent of acquisition rate providing excellent compatibility with the flow rates used in ultra-high-performance liquid chromatography and capillary electrophoresis Resolving power constant across mass range High sensitivity (superior to triple quadrupole in full scan mode – no loss of ions associated with "scanning" technologies) Mass range of the analyser is unlimited in principle, as this is based on the time it takes the ion of interest to reach the detector Parallel detection of all the ions over the complete mass range is relatively straightforward Cheaper than orbitrap	Medium-to-high resolving power Ion source and detector limit mass range
Orbitrap	Superior resolving power than TOF at slow scan speeds (resolving power is inversely proportional to scan speed) High sensitivity (superior to triple quadrupole in full scan mode – no loss of ions associated with "scanning" technologies)	Resolution decreases proportionately as mass increases More expensive than TOF

Table 1.7. Comparison between TOF and orbitrap analysis. Adapted from Wood, (2019).

1.11.1.1 Theory of TOF

The concept of TOF was first described in the 1940's (Stephens *et al.,* 1946) and it is based on determining the *m/z* by measuring the time it takes an ion to travel over a fixed distance (*i.e.* from ion pulser plate to the detector via a field free flight tube and reflectron). For a comprehensive mathematical description of the theory of TOF see Boesl, (2017). In brief this can be simplified and denoted by equations 1-6, see Figure 1.10.

$$E_k = \frac{mv^2}{2} = qVs = zeVs = E_{el} \quad \text{(Equation 1)}$$

$$v = \left(\frac{2zeVs}{m}\right)^{\frac{1}{2}} \quad \text{(Equation 2)}$$

$$t^2 = \frac{L}{v} \quad \text{(Equation 3)}$$

$$t^2 = \frac{m}{z}\left(\frac{L^2}{2evs}\right) \quad \text{(Equation 4)}$$

$$\left(\frac{m}{z}\right)^{\frac{1}{2}} = \left(\frac{\sqrt{2eVs}}{L}\right)t \quad \text{(Equation 5)}$$

$$^m/_z = A + B^2 \quad \text{(Equation 6)}$$

Figure 1.10. Mathematical denotation of TOF. E_k – kinetic energy; m – mass; v – velocity; q – electric charge; v_s – accelerating voltage; e – elementary charge constant; E_{el} – potential energy; z – charge number; t – time; L – distance; A – constant encompassing terms L and v_s; B – constant encompassing a corrected time zero as measured time zero may not correspond to true time zero. Adapted from de Hoffmann, (2007).

Equation 1 denotes the relationship between an ion with mass m and total charge $q = ze$ which is accelerated by accelerating voltage v_s, essentially this shows that electrical potential energy E_{el} is converted to kinetic energy E_k. Equation 2 denotes the velocity of the ion leaving the ion pulser plate. After ions are accelerated into the field free flight tube they travel at a constant velocity over a fixed distance (via a reflectron) to the detector. The time t required to cover this distance L is denoted in equation 3. Replacing v by its original value gives the denotation on equation 4, which combines the terms relating to time and energy required to accelerate ions. As the goal of analysis is to determine the *m/z* it is important to rearrange equation 4 to make this the subject as per equation 5. This equation can be simplified to what is denoted in equation 6 as distance L and accelerating voltage vs are constant for a given TOF instrument, and the relationship between $m^{1/2}$ and t is linear, thus can be simplified into a constant, A. A second constant B

can be added to correct for time delays as the measured time zero will not correspond to the true time zero due to (1) the time the control electronics send a start pulse to the time that high voltage is present on the rear ion pulsar plate and (2) the time an ion reaches the front of the ion detector plate surface until the signal generated by the ion is digitised by the acquisition electronics.

Figure 1.11 shows the schematic representation of the Agilent 6550 QTOF-MS utilised in the work presented herein. The beginning of the analytical process in this system starts with the introduction of the liquid chromatography (LC) eluent (containing the sample) into the spray chamber via a nebulizer into the MS where ions undergo electrospray ionisation (ESI). For mechanistic aspects of ESI see Bruins (1998). In brief, ionic species are transferred from solution into gaseous phase. In the Agilent 6550 system (Agilent 6200 Series TOF and 6500 Series Q-TOF LC/MS System Concepts Guide, 2014) this begins as LC eluent is sprayed at atmospheric pressure through the nebuliser (held at ground state) at a right angle to the capillary, which is raised to a high voltage. The spray is thermally focused by the dual jet spray technology (heated nitrogen gas). This together with the strong electrostatic field generated from the voltage applied to the capillary, causes the LC eluent to break up into small droplets and the preferential migration of ions of one polarity to the droplet surface.

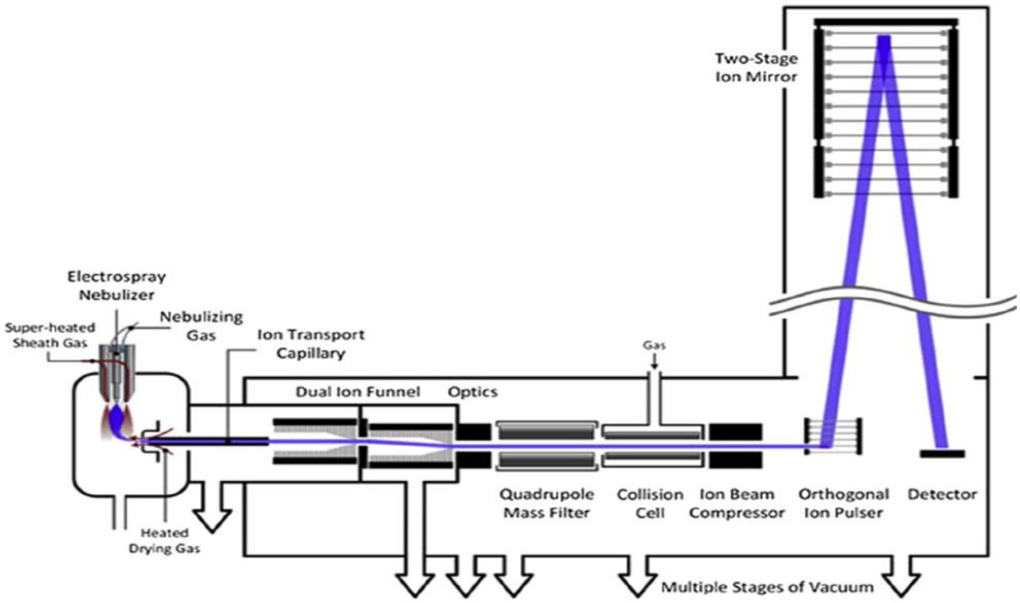

Figure 1.11. Schematic of Agilent 6550 quadrupole TOF mass spectrometer (García-Reyes *et al.,* 2017).

As the heated nitrogen gas is sprayed solvent evaporation starts to occur resulting in the formation of smaller droplet sizes forcing the predominantly like surface-charges closer together. This process continues until coulombic repulsion equals the surface tension of the droplet and the droplet explodes to produce smaller droplets, which are subject to further evaporation. This occurs until the charge density reaches the critical limit of ~10^8 V/cm^3, and ion evaporation occurs. The ejected ions are then drawn into the hexabore capillary (held at ~4 kV) and transferred into the dual ion funnels, which remove excess gas and neutral species and captures charged species. Ions are initially transmitted into the first funnel held at high pressure (7-14 Torr), and then into the second funnel held at a lower pressure (1-3 Torr). Radio frequency (RF) and direct current voltages are applied to the funnels to focus the ions to the centre of the funnel and accelerate them towards the exit and the octopole, respectively. This region of the mass spectrometer exists under high vacuum. Ions are focused by the octopole due to a RF voltage applied to the parallel rods that repel ions above a particular mass range toward the centre of the rod set. Ions then progress through focusing lenses into the

quadrupole mass filter, which optimises ion transmission and spectral resolution through application of RF frequency, and into the hexapole collision cell containing nitrogen (only active during fragmentation experiments when a collision energy voltage is applied over the accelerating linear voltage to generate fragments or product ions).

Ions are then focused via an ion beam compressor (octopole) to enter the ion transfer region which contains an orthogonal ion pulser which consists of a stack of ion plates. As ions progress into this stack with vertical and horizontal momentum a high voltage potential is applied to the back plate to accelerate ions through the stack towards the detector. Ions travel through the flight tube (field free) towards a 2-stage mirror called a reflectron. This enables the refocusing of ions, and the effective doubling of the distance ions travel.

As ions reach the detector they will strike its surface which contains a series of small tubes that span from the front to the rear of the plate. As ions strike they cause electrons to be released and start a process of electrical signal amplification (10 times more electrons leave the plate than enter). As electrons leave the microchannel plate they are focused onto a scintillator, which produces a photon of light. This is then focused through 2 small lenses onto a photomultiplier tube, which produces the electrical signal read by the data system allowing the calculation of exact mass.

1.11.2 Hyphenated techniques used with HRAMS

The use of hyphenated techniques with HRAMS is commonplace and provides an additional dimension of specificity to analysis. This is a vast area and as such the brief focus will be on the approaches used in the work presented herein, specifically LC for liquid biological samples and DESI for tissue imaging.

1.11.2.1 Liquid chromatography

LC is central to metabolomic experiments for the analysis of biological material including serum (Dunn *et al.,* 2011), urine (Want *et al.,* 2010) and

cell culture media (Want *et al.,* 2013). Common to all of these approaches is the use of reversed and normal phase chromatography. Using this common 2-pronged strategy facilitates the maximum coverage of non-polar and polar metabolites, respectively. This is because LC delays the transit of metabolites into the mass spectrometer, and provides an orthogonal mechanism of separation. This is vitally important when trying to identify a metabolite, especially when the compound is isomeric. Typical chromatographic conditions used for the analysis of serum (Dunn *et al.,* 2011) and urine (Want *et al.,* 2010) are:

1. *Reversed phase chromatography* - conditions include water and methanol as a mobile phase pair, containing a buffer (*e.g.* ammonium formate) and a C18 analytical column. This chromatographic approach is for the separation of less polar compounds.

2. *Normal phase chromatography* - conditions include water and acetonitrile as a mobile phase pair, containing a buffer (*e.g.* ammonium formate) and a hydrophobic interaction liquid chromatography (HILIC) column. This chromatographic approach is for the separation of polar compounds.

Both approaches tend to utilise analytical columns with a sub 2 μm particle size operated under high pressure facilitating improved resolution and peak capacity.

1.11.2.2 Mass spectrometry imaging

Mass spectrometry imaging (MSI) is a unique approach to the analysis of complex matrices (*e.g.* tissue) that provides both chemical and spatial information, which can be processed to generate chemical images (Wu *et al.,* 2013; Perez *et al.,* 2019). Ambient ionisation is a subfield of MSI that enables the analysis of a native sample under atmospheric conditions with minimal or no sample preparation; ionisation occurs outside of the mass spectrometer before ions are drawn into the mass spectrometer for analysis (Figure 1.12). The major benefit of this approach is that it facilitates soft desorption and

ionization of analytes, and enables more rapid analysis owing to minimal or no sample pre-treatment (Perez *et al.,* 2019). There are many variants of MSI; the work presented herein (Chapter 7) utilised DESI coupled to HRAMS for the analysis of neurotransmitter metabolites, which has the capability of generating imaging resolutions of 100-200 μm (Perez *et al.,* 2019), more recently Shariatgorji *et al.* (2019) published on the comprehensive mapping of neurotransmitter networks using matrix-assisted laser desorption ionization (MALDI) coupled to HRAMS. This particular approach employs a fluoromethylpyridinium based reactive matrix to facilitate the covalent charge tagging of molecules containing primary or secondary amine groups enabling a lateral resolution of 10 μm.

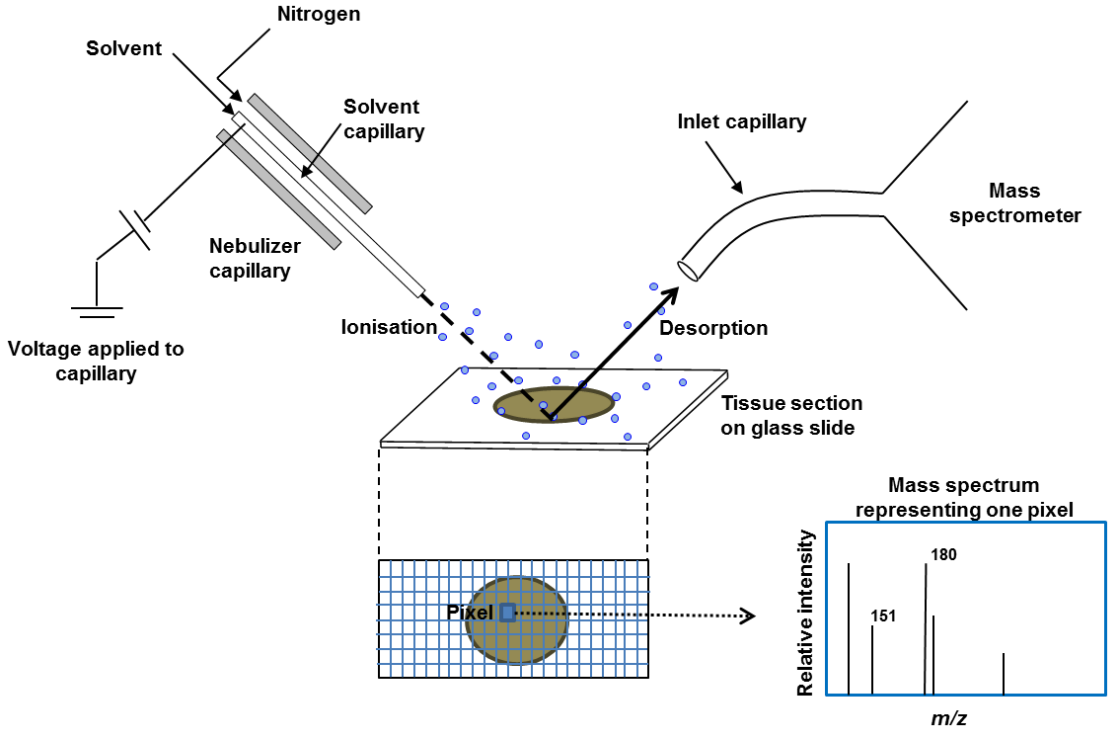

Figure 1.12. Desorption electrospray ionization. Adapted from Shariatgorji *et al.* (2014) and Perez *et al.* (2019).

1.11.3 NMR

Proton (^{1}H) NMR can provide information on the structure and energetics of a molecule (metabolite), and has been utilised in the field of metabolomics for over 50 years (Wishart, 2019). The work presented herein utilised ^{1}H NMR as a complementary technique to HRAMS, and it exploits the magnetic properties of a proton (nuclear spin) (Bothwell *et al.,* 2011), which are very sensitive to the surroundings in which they weakly interact (Hore, 2017). It is these properties that make NMR highly suited to understanding the atomic structure of a molecule. NMR spectra from molecules in a liquid can reveal 5 important pieces of information (Hore, 2017):

1. The intensities of individual resonances (influenced by the number of nuclei responsible).
2. Chemical shifts - interaction of nuclear spins with an applied magnetic field.
3. Spin-spin coupling - interaction with one another.
4. Spin relaxation - restoration of thermal equilibration.
5. Chemical exchange - effects of conformation and chemical equilibria.

This information is obtained by placing a sample into a strong magnetic field (Figure 1.13) and exposing it to electromagnetic radiation in the form of RF pulses. The latter excites protons (depending on the structure of the molecule), and as they relax back to their unexcited state the energy emitted is recorded as an oscillating electromagnetic signal, referred to as free induction decay (FID). This represents a complex wave function (intensity versus time) that requires fourier transformation (Ernst *et al.,* 1966) to generate a spectrum of intensity versus frequency. Data in the NMR spectrum are represented as a series of peaks with chemical shift (δ), in parts per million (ppm), on the x-axis and intensity along the y-axis. The chemical shift is the resonant frequency of the specific nucleus compared to the nucleus of an internal standard, typically tetramethylsilane (TMS). The distance between the resonant frequency observed and the TMS signal depends on the chemical environment of the proton (*i.e.* the molecular structure). Protons that exist in different parts of the molecule have a different

chemical shift which gives rise to a unique pattern of peaks with respect to chemical shift and peak intensity. In certain sample types, for example serum TMS is not used as an internal standard due to interference from high protein concentrations (Bell *et al.,* 1988; Nicholson *et al.,* 1989; Chatham *et al.,* 1999; Nagana *et al.,* 2014). In this situation spectra can be aligned to a metabolite always present in biological samples, for example glucose or lactate.

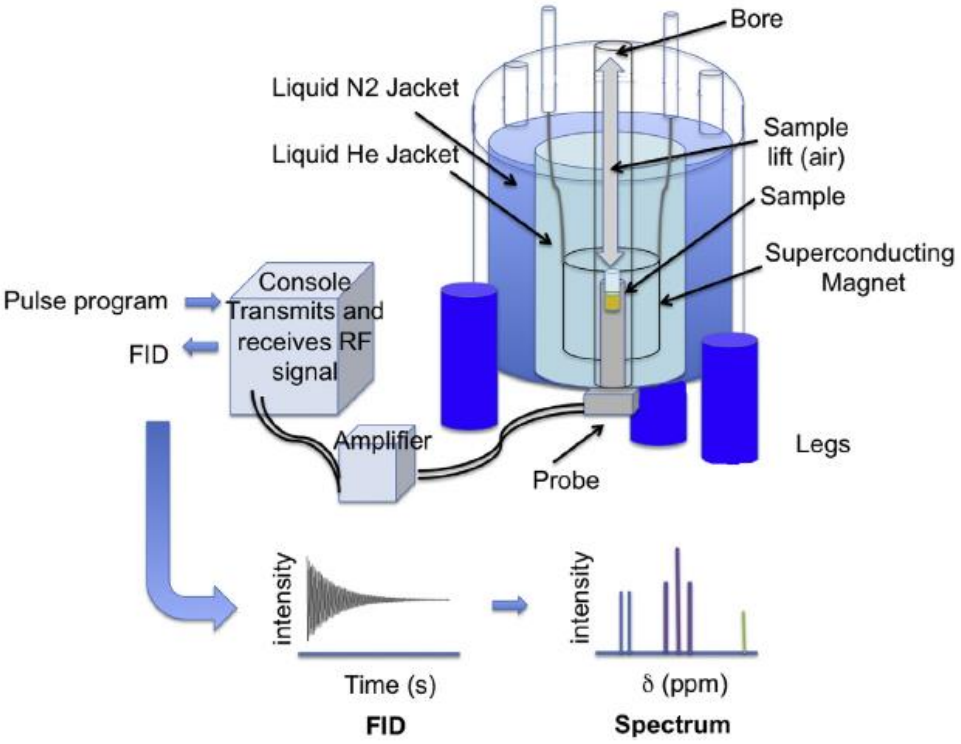

Figure 1.13. Schematic representation of a ^{1}H NMR spectrometer (Rankin *et al.,* 2014). This shows a superconducting magnet in its centre. This is kept at 4 K by a sleeve of liquid helium – evaporation of this is prevented by a vacuum and a jacket containing liquid nitrogen. The RF coil contained in the sample probe sits in the bottom of the magnet within its bore. Sample analysis is performed within the NMR tube; here the superconducting magnet (*e.g.* 16.5 tesla, 700 MHz – Bruker Advance III) causes protons to spin. The RF coil sends a RF pulse sequence to excite them and collects the free-induction decay as they relax back to equilibrium. Pulse sequences are programmed using a computer and sent to the console,

which acts both as a RF transmitter and receiver. The signals are amplified on transmission and receipt. The FIDs are fourier transformed to produce ^{1}H NMR spectra of intensity versus chemical shift (δ), typically between 0-10 ppm. Figure 1.14 shows ^{1}H nuclei chemical shifts due to different chemical environments.

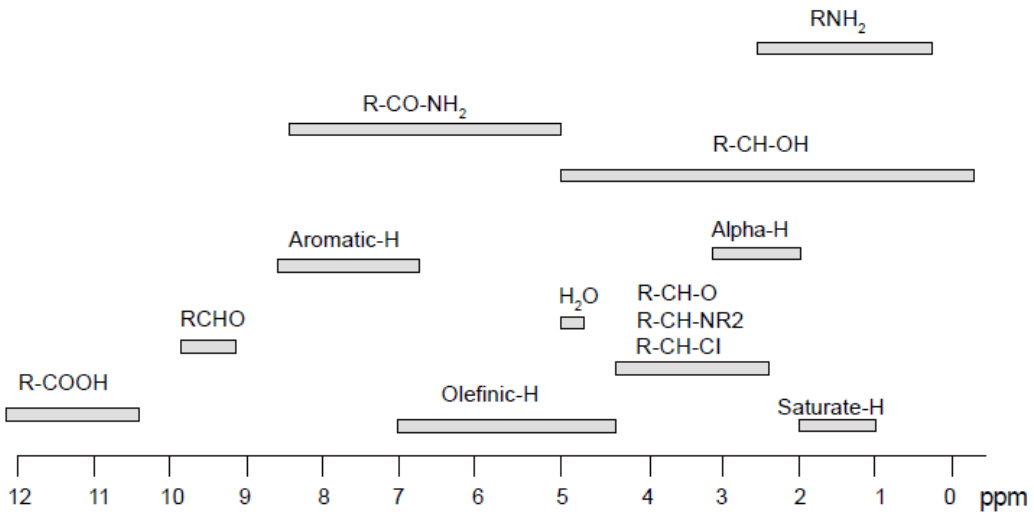

Figure 1.14. ^{1}H nuclei chemical shifts due to different chemical environments (Fuloria *et al.,* 2012).

Typically the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence is employed to identify smaller metabolites in the presence of more complex structures like proteins and lipoproteins (Meiboom *et al.,* 1958). This is possible because there is a long delay between the excitatory pulse and the acquisition period. This means larger molecules (*e.g.* lipids and proteins) will have stopped spinning, when smaller molecules are still spinning producing a FID signal. The mathematical principles that underpin ^{1}H NMR are complex, for details see Hore, (2017).

1.12 Challenges in metabolomic studies

A major challenge in NMR or HRAMS based metabolomic experiments is mining information from the vast volume of experimental data generated, and integrating it into a meaningful biological context (Goodacre, 2005; Barupal *et al.,* 2018). Much of the data generated are redundant, in part this is a reflection of signals that are generated from entities that are not present in the sample (*i.e.* contaminants) and originate from sample collection and preparation processes (Table 1.8). Data redundancy in the context of metabolomics also relates to the high degree of related signals within a data set (Chen *et al.,* 2017). For example, raw unprocessed LC-MS data includes *m/z* retention time pairs not only for the native entity, but also related signals representing the same entity (*i.e.* associated isotopes and adducts). It is essential therefore that sample collection, preparation and analysis, as well as data processing are rigorous and reproducible. There are a number of factors that can influence this which are summarised in Table 1.9.

Huge efforts are being made to standardise study design, which is complex (Jacyna *et al.,* 2019), and the analysis of raw data (Ren *et al.,* 2015). Data sharing is also strongly encouraged (Considine *et al.,* 2019) in the metabolomics community, and vast progress in this area can be seen by the number of entries in the Human Metabolome Database (~115 000) (Wishart *et al.,* 2018). Moreover, there is an increasing availability of spectral data for small molecules in public libraries like METLIN, KEGG, MIST-MS, ChemSpider, LipidMaps and PubChem (Milman *et al.,* 2016). The major limitation to all of these is that data are generated using a variety of analytical platforms with different configurations (*e.g.* different LC and mass spectrometer source parameters) so are not entirely transferable. For this reason, there are benefits in establishing in-house metabolite libraries that relate to known analytical conditions (Norman *et al.,* 2019a).

Experimental step	Confounder	Potential solution	Reference
Experimental design	Confounding factors (*e.g.* gender, genetic make-up, age, diet, lifestyle) Power study to ensure enough samples per group Analytical greater than biological variability	Control and limit confounding factors; admission to clinic to restrict environmental influences Appropriate number of samples based on pilot study; biological replicates Regular injection of QCs, blanks, labelled IS; sample randomisation	Kirwan *et al.,* 2018 Blaise *et al.,* 2016 Broadhurst *et al.,* 2018 Begou *et al.,* 2018 Beger, 2018
	Metabolome changes: – Arterial vs venous blood – Circadian rhythm – Pre- vs post-prandial status – Sample type/preservatives – Time between sampling and storage	Use protocols describing sampling procedures (matrix, sampling site, containers, storage conditions, time of day, fasting) Reproducible sampling conditions	Kohler *et al.,* 2016
Sample storage	Chemical and enzymatic degradation and/or interconversion, altering both qualitative/quantitative information	Immediate quenching (*e.g.* 60–100 % MeOH) and snap-freezing in liquid nitrogen Sample storage in adequate containers at -80 °C, preferably up to 6 months; 3 freeze–thaw cycles maximum Use preservatives if needed	Kohler *et al.,* 2016 Kirwan *et al.,* 2018

Sample preparation	Analytical errors (poor repeatability, degradation, contamination) Between-batch variation (large-scale studies)	Automate sample preparation; use labelled IS; work in temperature regulated rooms Single batch preparation of samples or division of samples into several batches, use of QCs	Kohler *et al.,* 2016
	Metabolite coverage	Keep the sample preparation as simple as possible but still fit-for-purpose	Beckonert *et al.,* 2007 Want *et al.,* 2010 Dunn *et al.,* 2011 Want *et al.,* 2013
Separation	RPLC: poor retention of polar compounds HILIC: poor repeatability; large peak shape	RPLC: use HILIC or CE for polar compounds HILIC: adequate equilibration; reproducible buffers; high proportion of ACN in sample	Want *et al.,* 2010 Dunn *et al.,* 2011 Want *et al.,* 2013
Ionisation	ESI: matrix effects (suppression/enhancement)	ESI: systematic evaluation of matrix effects during method development; use IS	Matuszewski *et al.,* 1998 Annesley, 2003 Chamberlain *et al.,* 2019
Detection	*Mass spectrometry:* Stereoisomers: same *m/z* and fragmentation patterns	Stereoisomers: use ion mobility spectrometry; chiral stationary phase	Kohler *et al.,* 2012
	NMR: Low sensitivity, relatively high sample volume Chemical shifts due to pH differences	Use of strong superconductive magnets and cryoprobes; microtubes Addition of buffer; post analysis alignment	Beckonert *et al.,* 2007 Kohler *et al.,* 2016

	Challenging peak identification	Simultaneous recording of 2D-J resolved spectra; apply STOCSY	
Data analysis	Selection of the most adequate data pre-processing and pre-treatment algorithms	PQN normalization and UV scaling; systematic data alignment; careful peak picking (adequate S:N ratio, peak width, number of features)	Kohl *et al.,* 2012 Ren *et al.,* 2015 Kohler *et al.,* 2016
	Misleading class separation in PCA and PLS-DA modelling	Systematic use of internal cross-validation methods	
	Confidence in metabolite identification and biological interpretation	MSI guidelines (see Table 1.10); fusion with other omics data; biomarker validation	Sumner *et al.,* 2007
Biomarker discovery	Biomarker validation	Validation criteria based on predictive, prognosis, or diagnostic purpose	Kohler *et al.,* 2016 Barupal *et al.,* 2018
	Clinical utility and application of novel biomarkers	Fit for purpose validation (internal and external, clinical trials, continued surveillance)	

Table 1.8. Summary of potential confounding factors in a clinical metabolomics experiment/study and potential solutions.
IS – internal standard; MSI – metabolomics standards initiative; PQN – probabilistic quotient normalisation; RPLC – reversed phase liquid chromatography; HILIC – hydrophobic interaction liquid chromatography; MeOH – methanol; ACN – acetonitrile; S:N – signal to noise; ESI – electrospray ionisation; CE – capillary electrophoresis; NMR – nuclear magnetic resonance; PCA – principal component analysis; PLS-DA – partial least squares discriminant analysis; STOCSY – statistical total correlation spectroscopy; QC – quality control; UV – unit variance. Adapted from Kohler *et al.* (2016).

In addition, efforts have been made to standardise the reporting of metabolite identification based on levels of evidence (Table 1.9).

Level of confidence	Description	Requisite analytical data
1	'Identified metabolites'	Two orthogonal analytical techniques applied to the analysis of both the metabolite of interest and to a chemical reference standard of suspected structural equivalence, with all analyses performed under identical analytical conditions within the same laboratory. Examples of appropriate orthogonal data: (1) accurate mass via MS with retention time (2) accurate mass MS and fragmentation data or isotopic pattern (3) 2D NMR spectra, full ^{1}H and/or ^{13}C NMR spectra
2	'Putatively annotated compounds'	As for levels 3 and 4, including spectral (NMR and/or MS) similarity with public or commercial libraries
3	'Putatively characterised compound classes'	As for level 4, plus spectral and/or physicochemical properties consistent with a particular class of organic compounds
4	'Unknown'	A discernible spectral signal (NMR, MS or other) that can be reproducibly detected and quantified

Table 1 9. Proposed minimum reporting standards for chemical analysis (Sumner *et al*., 2007). NMR – nuclear magnetic resonance; MS – mass spectrometry.

1.13 Metabolomics and alkaptonuria

Gertsman *et al.* (2015a) was the first to report on the use of HRAMS to evaluate the serum metabolome of patients with AKU taking nitisinone (2-8 mg daily) over a period of 6 months to 3.5 years. This small study revealed the expected decrease in HGA and increase in tyrosine following treatment with nitisinone. This untargeted analysis also revealed significant increases in N-acetyl-L-tyrosine and γ-glutamyltyrosine, which is not surprising due to the significant hypertyrosinaemia that is observed following treatment with nitisinone. In a related publication (Gertsman *et al.,* 2015b) novel disturbances in tryptophan metabolism following treatment with nitisinone were also reported. Specifically, indole-carboxyaldehyde, indole-3-pyruvate and indole-3-lactate (I-3-L) were shown to increase 5-fold. It was proposed the tyrosine metabolite HPPA, which increases significantly following nitisinone therapy, up regulates tryptophan aminotransferase activity resulting in downstream changes in the concentration of tryptophan metabolites and not tryptophan itself. The significance of this is unknown, it has been suggested that aromatic keto acids increase the affinity of tryptophan transaminase for tryptophan (Lees *et al.,* 1973), thus altering its metabolism.

Davison *et al.* (2019b) also reported increases in serum N-acetyl-L-tyrosine and γ-glutamyltyrosine following treatment with nitisinone using HRAMS, but interestingly only observed a change in I-3-L. This study did show increases in other tryptophan metabolites including trigonelline and quinoline carboxylic acid. Novel changes were also observed in metabolites relating to the citric acid cycle (decrease in succinate and α-ketoglutarate) and xanthine metabolism (decrease in uridine and inosine). It was suggested that these changes along with those observed in N-acetyl-L-tyrosine and γ-glutamyltyrosine relate to changes in the redox state of the cell following treatment with the HGA lowering agent nitisinone.

Increases in 4-hydroxyphenylacetic acid and 4-hydroxybenzaldehyde, and a decrease in benzaldehyde were also reported following nitisinone treatment. The significance of the latter 2 metabolites is uncertain, but was suggested that they relate to the ochronotic pigment observed in AKU. The increase in 4-hydroxyphenylacetic acid is thought to result from increased activity of gut microbiota following treatment with nitisinone due to the presence of less oxidative stress.

Norman *et al.* (2019a) has also reported novel changes in urinary tryptophan and related metabolites in patients with AKU treated with nitisinone. In this study urinary indoxyl sulphate, tryptophan and kynurenine decreased whilst xanthurenic acid increased following treatment. This study supports the changes reported in 4-hydroxybenzaldehyde and 4-hydroxyphenylacetic acid (Davison *et al.,* 2019b), but also reported novel changes in 4-coumarate, tyramine, mandelic acid and phenylacetic acid. Interestingly a number of changes were also observed in purine metabolism. Importantly many of these changes reported in patients were also observed in a murine model of AKU treated with nitisinone that was used in this study reinforcing that the changes observed were a consequence of nitisinone therapy.

1.14 Aims of research

There were 2 main aims to the research presented in this thesis. The first was to establish a methodological approach for the comprehensive evaluation of the metabolome in serum and urine samples obtained from patients with AKU, before and after treatment with nitisinone. The rationale for this is that the knowledge of the impact of nitisinone treatment on the metabolome is very limited; published literature has focused on a limited number of metabolites, primarily HGA and tyrosine. A better understanding of the wider metabolic consequences of nitisinone therapy is essential for its use in the treatment of AKU, particularly in relation to neurotransmitter metabolism, oxidative stress and ochronosis. In this thesis a comprehensive targeted metabolomic profiling strategy using LC-QTOF-MS is presented and

applied for the analysis of urine (Chapter 2) and serum (Chapter 3) from patients with AKU treated with nitisinone. Additionally, in Chapter 3 a methodological approach utilising NMR for the evaluation of the serum metabolome is presented.

The second aim was more specific, and focused on whether nitisinone-induced hypertyrosinaemia alters neurotransmitter metabolism and the metabolism of other amino acids. Whilst changes to neurotransmitter and amino metabolism were evaluated when performing comprehensive targeted metabolomic profiling using LC-QTOF-MS for the analysis of urine (Chapter 2) and serum (Chapter 3) data acquired were qualitative, and no internal standards were used. As such additional targeted quantitative analysis of urinary neurotransmitter metabolites was performed using liquid chromatography tandem mass spectrometry in samples collected from patients following treatment with nitisinone (Chapters 5 and 6). Quantitative analysis of serum amino acids using liquid chromatography (Chapter 4) was also performed to determine if amino acid metabolism beyond that of tyrosine was affected by treatment with nitisinone. Data from the analysis of urinary neurotransmitter metabolites and serum aromatic amino acids were also correlated with psychometric data (BDI-II Scores, Chapter 6) collected from patients to assess if changes in biochemical parameters could be related to changes in mood in patients following treatment with nitisinone.

Due to the limitations of measuring neurotransmitters and their related metabolites in serum and urine, and the subjective nature of psychometric data analysis; patterns of neurotransmitters were also assessed directly in brain tissue from a murine model of AKU following treatment with nitisinone (Chapter 7). Analysis of CSF from the same murine model of AKU following treatment with nitisinone was also performed using LC-QTOF-MS to provide a direct assessment of neurotransmitter metabolism in the CNS (Chapter 8).

The integration of biochemical and psychometric data from human subjects, and biochemical data from the murine model of AKU following nitisinone therapy has provided a new insight into the impact of nitisinone therapy on

the serum and urine metabolome in AKU, and more specifically the impact of nitisinone induced hypertyrosinaemia on neurotransmitter metabolism.

1.15 Declaration and acknowledgements

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Part II

Assessment of the impact of nitisinone therapy on the serum and urine metabolome in patients with alkaptonuria

Chapter 2

A comprehensive LC-QTOF-MS metabolic phenotyping strategy: application to alkaptonuria

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2.1 Abstract

Background: Identification of unknown chemical entities is a major challenge in metabolomics. To address this challenge, we developed a comprehensive targeted profiling strategy, combining 3 complementary liquid chromatography (LC) quadrupole time-of-flight mass spectrometry (QTOF-MS) techniques and in-house accurate mass retention time (AMRT) databases established from commercial standards. This strategy was used to evaluate the effect of nitisinone on the urinary metabolome of patients and mice with alkaptonuria (AKU). As hypertyrosinaemia is a known consequence of nitisinone therapy, we investigated the wider metabolic consequences beyond this.

Materials and methods: 619 standards (molecular weight 45-1354 Da) covering a range of primary metabolic pathways were analyzed using 3 LC methods, 2 reversed phase and 1 normal phase, coupled to QTOF-MS. Separate AMRT databases were generated for the 3 methods, comprising chemical name, formula, theoretical accurate mass and measured retention time. Databases were used to identify chemical entities acquired from non-targeted analysis of AKU urine; match window theoretical accurate mass ±10 ppm and retention time ±0.3 min.

Results: Application of the AMRT databases to data acquired from analysis of urine from 25 patients with AKU (pre-treatment and after 3, 12 and 24 months on nitisinone) and 18 *HGD*$^{-/-}$ mice (pre-treatment and after 1 week on nitisinone) revealed 31 previously unreported statistically significant changes in metabolite patterns and abundance, indicating alterations to tyrosine, tryptophan and purine metabolism post-nitisinone.

Conclusions: The comprehensive targeted profiling strategy described here has the potential of enabling discovery of novel pathways associated with pathogenesis and treatment of AKU.

2.2 Introduction

Metabolic profiling has potential to advance knowledge of disease beyond established biochemical pathways and will play a major role in precision medicine (Fiehn *et al.,* 2002; Holmes *et al.,* 2008; Baker *et al.,* 2011; Nicholson *et al.,* 2012; Beger *et al.,* 2016). However, metabolite identification is a major challenge in untargeted profiling studies using mass spectrometry (MS) (Wishart *et al.,* 2011; Dunn *et al.,* 2013) since they produce many chemical signals representing 'unknowns'. To address this challenge, we generated 3 accurate mass/retention time (AMRT) databases from 619 metabolite standards using liquid chromatography-quadrupole time-of-flight-mass spectrometry (LC-QTOF-MS). We applied this strategy to the inborn error of metabolism alkaptonuria (AKU, OMIM 203500). In AKU, bi-allelic mutations in the homogentisate 1,2-dioxygenase (*HGD*) gene result in a lack of homogentisate 1,2-dioxygenase (HGD, E.C.1.12.11.5) (Zatkova *et al.,* 2011), leading to increased homogentisic acid (HGA), a metabolite of tyrosine catabolism (Figure 2.1). HGA accumulates in connective tissue, particularly cartilage, where it is deposited as a dark pigment, a process termed ochronosis. Ochronosis underlies a range of clinical features in AKU, the most debilitating of which is severe, early-onset osteoarthropathy that alters the physico-mechanical properties of cartilage (Taylor *et al.,* 2011; Ranganath *et al.,* 2013).

Nitisinone has emerged as a promising therapeutic agent in AKU because it reduces circulating HGA concentrations (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al*., 2016; Milan *et al.,* 2017) by reversibly inhibiting 4-hydroxyphenylpyruvate dioxygenase (E.C. 1.13.11.27). Nitisinone has been shown to completely inhibit ochronosis in an *HGD*$^{-/-}$ mouse model of AKU (Preston *et al.,* 2014). Although not currently licenced for AKU, nitisinone is being used to treat patients attending the National Alkaptonuria Centre (NAC) in the UK. One of the major metabolic consequences of nitisinone is hypertyrosinaemia (Lindstedt *et al.,* 1992; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; McKiernan *et al.,* 2015; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a;

Davison *et al.,* 2018b; Davison *et al.,* 2018c) (Figure 2.1). The objective of our study was to apply the developed profiling strategy to urine from patients with AKU and BALB/c (*HGD*$^{-/-}$) mice to understand the wider metabolic consequences of nitisinone treatment.

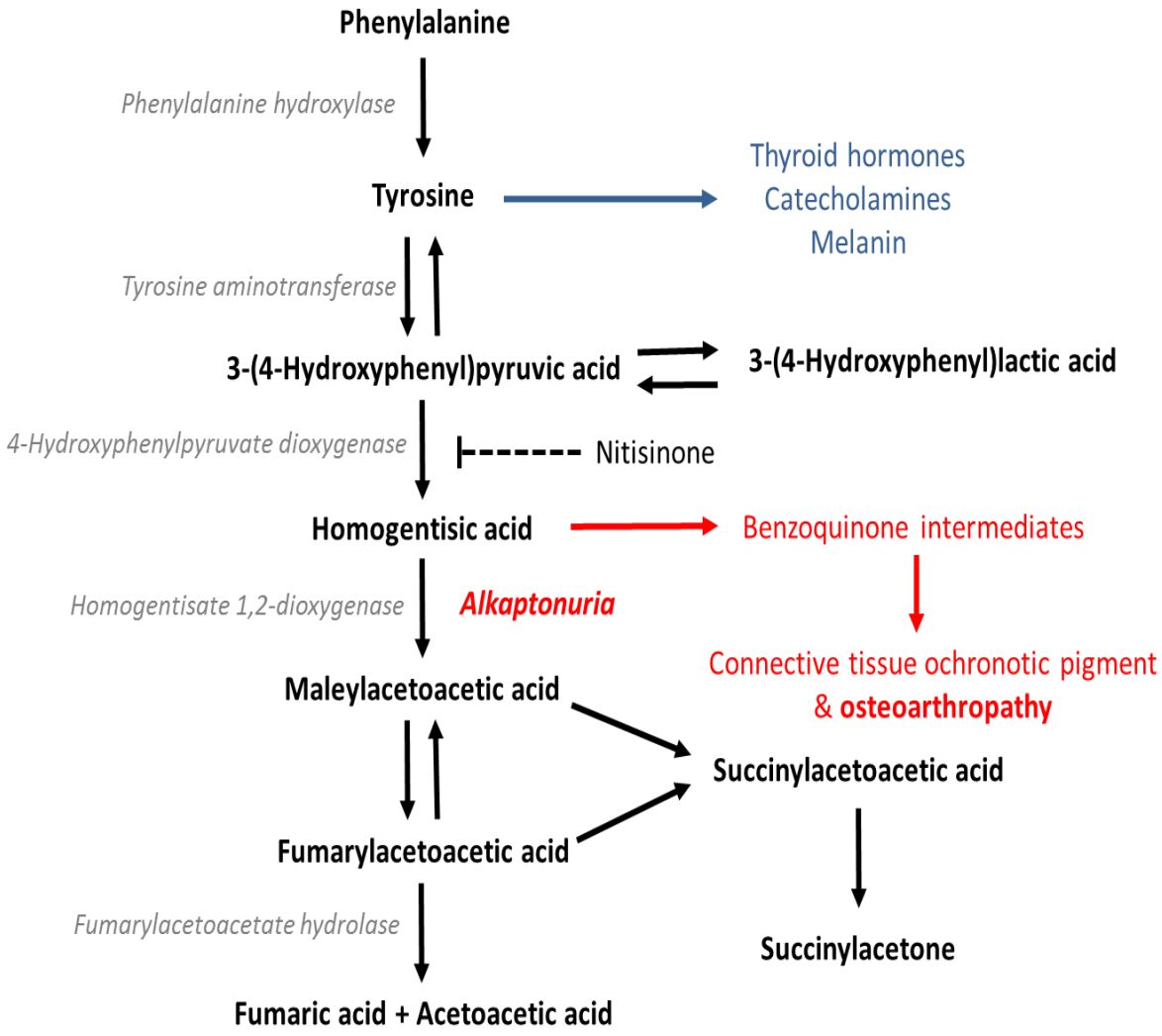


Figure 2.1.Tyrosine catabolic pathway with reference to its defect in alkaptonuria and treatment with nitisinone. Lack of the enzyme homogentisate 1,2-dioxygenase increases concentrations of circulating HGA by preventing its conversion to maleylacetoacetic acid. It is previously established that nitisinone has the upstream consequence of increased concentrations of tyrosine, 3-(4-hydroxyphenyl)pyruvic acid and 3-(4-

hydroxyphenyl)lactic acid as a result of the metabolic block induced by nitisinone.

2.3 Materials and methods

2.3.1 Metabolite standards library preparation

619 standards (IROA Technology MS metabolite library of standards; molecular weight 45-1354 Da, Supplementary Table 2.1) were purchased from Sigma-Aldrich (UK) in 7 deep well plates. Each compound was present at 5 μg/well (>95 % purity). The standards covered a broad range of primary and intermediary metabolism, including the following compound classes: carboxylic acids, amino acids, biogenic amines, polyamines, nucleotides, coenzymes, vitamins, carbohydrates, fatty acids, lipids, steroids and hormones (Supplementary Table 2.1).

Prior to analysis, plates were thawed (stored at -80 °C) at ambient temperature (18 °C) and compounds reconstituted in 15 μL methanol (LC/MS grade, Sigma-Aldrich) followed by 285 μL deionized water (DIRECT-Q 3UV Millipore water purification system). Plates 1-5 were left to stand for 1 h following addition of water and methanol. Plates 6 and 7 (primarily non-polar compounds) were left to stand for 2 h at room temperature following addition of methanol to ensure solubilisation; water was then added. Plates were agitated on a plate shaker (MTS 2/4m IKA, Germany) at 600 rpm for 10 min. Compounds were pooled across rows of each plate (12 wells/row). 20 μL was removed from each well and pooled into 1 well (12 compounds analyzed per injection). Fifty-six injections were performed in positive and negative polarity across all plates (112 injections for each LC-QTOF-MS method; Supplementary Figure 2.1). The total volume of each pool was 240 μL with each compound at 14 mg/dL (1-31 μmol/L).

2.3.2 Evaluation of LC-QTOF-MS strategy and effect of nitisinone on the metabolome using AKU urine

Acidified 24 h urine collections (2.5 L bottles containing 30 mL of 5N H_2SO_4) were obtained from 25 patients with AKU attending the NAC (12 male, mean(±SD) age 51±15 years). Samples were collected before nitisinone, then at 3 (2 mg nitisinone every other day), 12 and 24 (2 mg nitisinone daily) months and stored at -80 °C. Samples were diluted 1:3 with deionized water and stored at -80 °C as 3 separate aliquots for analysis by methods 1-3. Metabolomic analysis was part of the diagnostic service to patients being seen at the NAC and with approval from the Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (Audit no ACO3836).

Mouse urine (non-acidified) was from 18 (9 male, mean age 27±12 weeks) *HGD*$^{-/-}$ BALB/c mice (Preston *et al.,* 2014) bred from laboratory stocks at the University of Liverpool. Samples were obtained on a single-collection basis before treatment then after 1 week on nitisinone, administered in all drinking water (4 mg/L), supplied *ad libitum*. Mouse urine was collected onto cling film, pipetted into sample tubes and stored at -80 °C. Analysis was performed following dilution of 1:9 with deionized water. Mouse breeding and dosing was authorized under the Animals (Scientific Procedures) Act, UK.

Samples were pooled for quality assurance in profiling experiments. For each sampling time point, a representative pool was created by pooling
20 μL of each individual urine sample. An additional overall pool was created separately for human and mouse experiments by pooling equal proportions of the pooled urine groups detailed above. Pooled human and mouse samples were stored and treated as above prior to analysis.

2.3.3 Analytical conditions

2.3.3.1 Chemicals

Water for mobiles phases was purified as per section 2.3.1. Methanol, acetonitrile, isopropanol (Sigma-Aldrich), formic acid (Biosolve, Netherlands) and ammonium formate (Fisher Scientific, Germany) were LC/MS grade.

2.3.3.2 Equipment

Sample analysis was performed on an Agilent 1290 Infinity LC coupled to an Agilent 6550 QTOF-MS equipped with a dual AJS electrospray ionization source (Agilent, Cheadle, UK).

2.3.3.3 Chromatographic conditions

Three chromatographic methods were designed to separate different compound classes. Method 1: non-polar compounds. Method 2: a range of polar/non-polar compounds. Method 3: polar compounds. Metabolite standards were analyzed using all 3 LC-QTOF-MS methods.

Method 1

A Zorbax Eclipse Plus C_{18} column (2.1 x 100 mm, 1.8 μm, Agilent, Cheadle, UK) was maintained at 60 °C (flow rate 0.4 mL/min). Mobile phases were (A) water and (B) methanol both containing 5 mmol/L ammonium formate and 0.1 % formic acid. The elution gradient started at 5 % B at 0-1 min increasing linearly to 100 % by 12 min, held at 100 % B until 14 min, returning to 95 % A for 5 min.

Method 2

Method 2 employed the same conditions and elution gradient as method 1, but with an Atlantis dC_{18} column (3.0 x 100 mm, 3 μm, Waters, UK).

Method 3

A BEH amide column (3.0 x 150 mm, 1.7 μm, Waters) was maintained at 40 °C (flow rate 0.6 mL/min). Mobile phases were (A) water and (B) acetonitrile (both containing 0.1 % formic acid). The elution gradient started at 99 % B decreasing linearly to 30 % from 1-12 min, held at 30 % B until 12.6 min, returning to 99 % B for 3.4 min.

Sample injection volume was 1 μL for metabolite standards and human urine, and 2 μL for mouse urine. The autosampler was maintained at 4 °C and the needle was washed with a solution of water:methanol:isopropanol (45:45:10 v/v) between injections.

2.3.4 Design of urine metabolic profiling experiments

Human and mouse urine was analyzed separately. Human samples were analyzed batch-wise using all 3 methods, negative followed by positive polarity. Mouse samples were analyzed by method 2 only due to limited sample volume, in 1 batch comprising both polarities.

The analytical sequence of each profiling batch was designed according to published guidance (Vorkas *et al.,* 2015). Each run commenced with 20 replicate injections of the overall pooled sample to condition the system. The order of individual samples was randomized computationally. Pooled samples were interspersed throughout the analytical sequence, every 10th injection. Injections of each sample group pool and the overall pooled sample were also placed at the start (post-conditioning) and end of each analytical sequence.

2.3.5 Data acquisition and processing

Data acquisition and processing were performed using the MassHunter suite (Agilent, Cheadle, UK). Data were acquired with Data Acquisition (Build 06.00). Metabolite standards and urine samples were analyzed in both polarities, mass range 50-1700 on an Agilent 1290 Infinity LC coupled to an Agilent 6550 QTOF-MS equipped with a dual AJS electrospray ionization source (Agilent, Cheadle, UK). The mass spectrometer was tuned and calibrated according to protocols recommended by the manufacturer. Acquisition was performed in 2 GHz mode, positive and negative ionisation polarity and mass range 50-1700. The capillary voltage was 4000 V and fragmentor voltage 380 V. The desolvation gas temperature was 200 °C with flow rate at 15 L/min. The sheath gas temperature was 300 °C with flow rate at 12 L/min. The nebulizer pressure was 40 psi and nozzle voltage 1000 V (for positive and negative ionisation modes). The acquisition rate was 3 spectra/s.

A reference mass correction solution was prepared in 95:5 methanol:water containing 5 mmol/L purine (CAS No. 120-73-0), 100 mmol/L trifluoroacetic acid ammonium salt (TFA, CAS No. 3336-58-1) and 2.5 mmol/L hexakis(1H,

1H, 3H-tetrafluoropropoxy)phosphazine (HP-0921, CAS No. 58943-98-9) (Agilent, Cheadle, UK). The solution was continually infused at a flow rate of 0.5 mL/min by a separate isocratic pump for constant mass correction [positive ionisation: purine (*m/z* 121.0509), HP-0921 (*m/z* 922.0098); negative ionisation: TFA (*m/z* 112.9856), purine (*m/z* 119.0363), HP-0921 (HP-0921 + formate adduct: *m/z* 966.0007)].

Quality checks and processing of raw data were performed with Qualitative Analysis (Build 07.00) (See Supplementary Figure 2.2). Extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run. Binary pump pressure curves for injections across each analytical sequence were overlaid to check chromatographic reproducibility.

Compound signals were extracted from the standards data in Qualitative Analysis (Build 07.00) by molecular formula using the 'find by formula' algorithm; mass window of theoretical accurate mass (calculated from molecular formula) ±5 ppm. Allowed ion species were: H^+ and Na^+ and additionally NH_4^+ for methods 1 and 2 (positive polarity); and H^- and additionally CHO_2^- for methods 1 and 2 (negative polarity). Charge state range was 1-2. Dimers were allowed.

Separate AMRT databases were created for each method for compounds that were retained and detected using personal compound database and library (PCDL) Manager (Build 08.00).This included theoretical monoisotopic mass, retention time (RT), molecular formula and chemical name (see Supplementary Tables 2.2-2.4 for these data, except for molecular formula which can be found in the complete databases online (Norman *et al.,* 2019b)). METLIN metabolites accurate mass database (Build 07.00) was used as the basis for each AMRT database (contained entries for 30,232 small molecules; comprising chemical name, empirical formula, theoretical monoisotopic mass and where available deposited mass spectra).

Urine profiling data were mined for signals matching AMRT database compounds using 'targeted feature extraction' (Profinder, Build 08.00). This uses the molecular formulae to extract and group spectral signals (*i.e.* adducts, multimers and isotopes), corresponding to individual database compounds. Feature extraction window; theoretical accurate mass ±10 ppm and database RT ±0.3 min. Allowed ion species were the same as specified above.

2.3.6 Detection of non-AMRT database compounds in urine

Urine data were also mined using Profinder for compounds not included in generated AMRT databases, but of interest for their predicted role in AKU/nitisinone metabolism. These compounds were associated with (a) increased tyrosine: N-acetyl-L-tyrosine, γ-glutamyl-L-tyrosine and tyramine-sulfate; (b) ochronotic pigment: 2,5-dihydroxybenzaldehyde, hipposudoric acid and norhipposudoric acid; or (c) nitisinone metabolism: hydroxy-nitisinone, nitisinone and 2-nitro-4-trifluoromethylbenzoic acid. For non-AMRT compounds, the same RT (range <0.3 min) was required across samples. Compound identifications were based on theoretical accurate mass ±10 ppm.

2.3.7 Data quality control and statistical analyses

Several quality control (QC) filters were applied to AMRT matched entities from each profiling batch. First, entities were retained if observed in at least 2 samples per experimental group (*i.e.* sampling time point). Data files were then exported from Profinder and imported into Mass Profiler Professional (MPP, Build 14.5). 24 h urinary creatinine measurements (Jaffe alkaline picrate reaction, Roche Diagnostics, Germany) were used as an external scalar for individual human urine samples (average values across each sampling time point used for pooled samples). Quantitative measurement of creatinine was not possible in mouse samples due to small sample volumes; the signal identified as creatinine by matched AMRT was used as external scalar for each sample using the peak area of the ^{13}C $[M+H]^+$ ion (^{13}C because ^{12}C creatinine signal was saturated) calculated in MassHunter Quantitative analysis (Build 06.00).

Entities were then further filtered in MPP based on data from pooled samples from each experiment. Entities were retained if observed in 100 % of replicate injections for at least one sample group pool, and with peak area coefficient of variation (CV) <25 % across replicate injections of all sample group pools.

Statistical analyses were performed in MPP based on peak area. Human urine profiles at 3, 12 and 24 months (following nitisinone treatment) were compared with baseline (pre-nitisinone) by one-way repeated-measures ANOVA. Mouse urine profiles were compared pre-nitisinone and 1 week on nitisinone by paired t-test. Benjamini-Hochberg false-discovery rate adjustment was used in all statistical significance testing. Fold changes (FC's) were calculated based on raw peak area. Principal component analyses employing 4-component models were also performed on each filtered dataset.

2.4 Results

2.4.1 Analysis of metabolite standards

Signals representing the chemical formulae for standards in each injection were extracted from the data by theoretical accurate mass ±5 ppm. Only standards with RT >0.3 min after the column void volume were considered. 519/619 (83.8 %) of the standards were retained sufficiently to be detected by at least 1 method (Supplementary Tables 2.2-2.4 shows the matches obtained by methods 1-3). 116/619 (18.7 %) and 226/619 (36.5 %) of the compounds were retained sufficiently and detected by 1 or 2 methods only, respectively, demonstrating the utility of combining data from the 3 chromatographic methods (Figure 2.2 (A)). RT, accurate mass and charge state were entered into an AMRT metabolite database for each method for matching unknowns against.

Figure 2.2 (B-D) shows the mass/RT distribution for compounds detected by

each method and highlights differences in selectivity for compounds from 3 example compound groups with different chemical properties: carbohydrates, amino acids and lipids. Method 3 retained and separated highly polar compounds such as carbohydrates (20/26 detected, RT range 5-9.5 min).

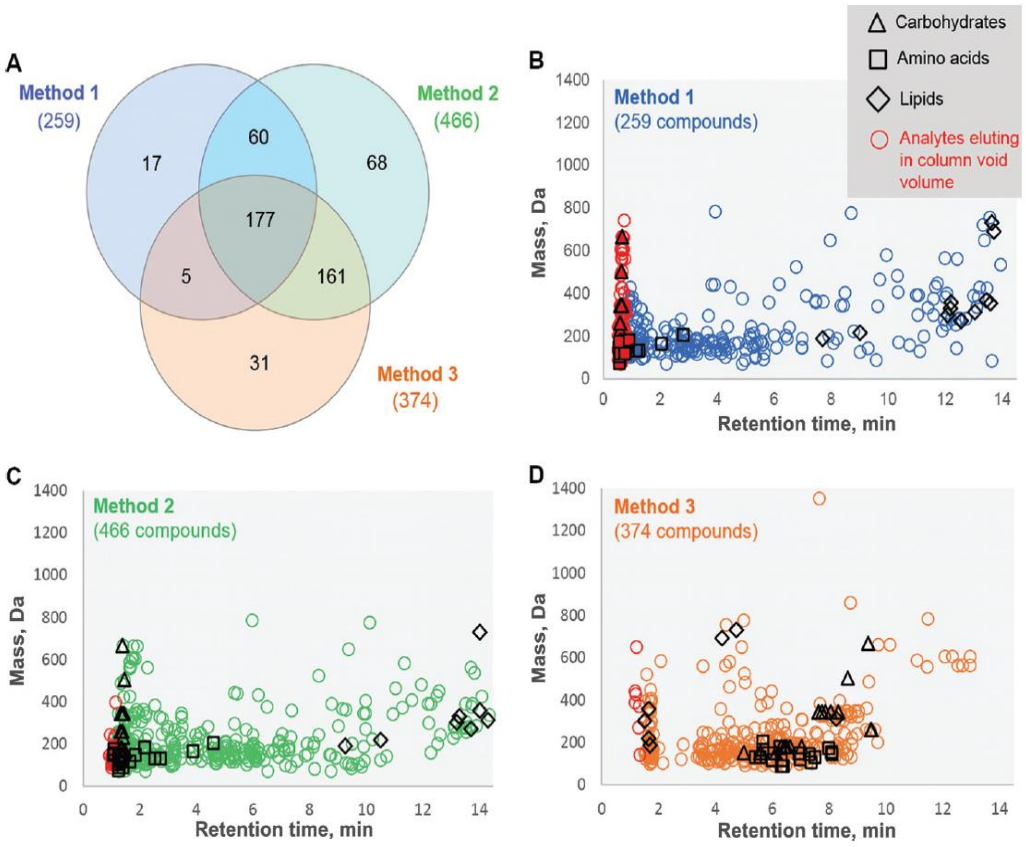


Figure 2.2. Summary of the data acquired from analysis of metabolite standards. (A) Venn diagram summarizing the number of compounds retained and detected by methods 1-3, both alone (non-overlapping sections) and in combination (overlapping sections). (B-D) Mass/RT scatter plots for metabolite standards detected with the 3 analytical methods, showing the different selectivities of the methods for compounds from different chemical classes.

In methods 1 and 2, the same carbohydrates were weakly retained; for method 1 all carbohydrates detected eluted within the initial column void

(24/26 detected, RT range 0.58-0.68 min) and for method 2 all carbohydrates detected eluted close to the initial void (23/26 detected, RT range 1.3-1.4 min). Method 1 showed some evidence of improved suitability for analysis of lipid-like compounds (11/11 detected, RT range 7.7-13.7 min) compared to methods 2 (8/11 detected, RT range 9.3-14.3 min) and 3 (7/11 detected, RT range 1.5-8.3 min), which detected fewer of these compounds within a narrower RT range overall. All 3 methods enabled detection of the amino acids, with method 3 appearing to show the most useful chromatographic resolution and retention (19/21 detected, RT range 5.4-8.1 min) compared to methods 1 (21/21 detected, RT range 0.6-2.8 min) and 2 (21/21 detected, RT range 1.0-4.6 min).

2.4.2 Identification of metabolites in pooled urine by AMRT

Unknown chemical entities were matched against the respective AMRT database generated for each method: accurate mass ±10 ppm, RT ±0.3 min. For data presented here, analysis comprised 2 replicate injections of the overall pooled urine from patients with AKU from the start and end of each analytical run (n=4). Only unknowns obtained from both injections in positive or negative polarity and with single AMRT compound matches were retained in this analysis. Additional QC filters were then applied to ensure reproducibility across the run: CV <25 % for peak area and RT between the 2 replicate injections. Table 2.1 summarizes matches retained for each method. No compounds were filtered out due to RT CV >25 %. The maximum RT CV percentage change between the replicate injections across all analytical runs (each comprised 205 injections) was 4.1 %.

Figure 2.3 (A) shows that 203 unique compound matches were obtained from urine. Fourteen AMRT matches were obtained by all 3 methods. An additional 61 AMRT matches were obtained by 2 methods, and 128 matches by only 1 of the methods, further supporting the increased coverage from the 3 methods in combination. Figure 2.3 (B-D) shows the mass/RT distribution of the AMRT match.

Feature extraction window	Filtering step	Number of urine AMRT matches					
		Method 1		Method 2		Method 3	
		+VE	-VE	+VE	-VE	+VE	-VE
Accurate mass 10 ppm, RT 0.3 min	None	274	153	248	105	111	74
	(1) Unique AMRT's only, manual curation	80	38	121	43	70	37
	(2) Abundance QC: peak area CV <25 % between replicates	75	36	107	35	59	30
	(3) RT shift QC: RT CV <25 % between replicates	75	36	107	35	59	30
Accurate mass 5 ppm, RT 0.15 min	Steps (1) - (3)	44	31	65	25	38	22
Accurate mass 2.5 ppm, RT 0.075 min	Steps (1) - (3)	17	21	22	12	14	13

Table 2.1. Number of accurate mass retention time (AMRT) matches obtained by the 3 methods and retained following quality control filtering and decreasing AMRT window size during feature extraction. RT, retention time; +VE – positive polarity; -VE – negative polarity

Table 2.1 and Supplementary Figure 2.3 show the number of AMRT matches obtained with narrower AMRT matching windows. For each method, >50 % of matches obtained with ±10 ppm and ±0.3 min post-QC remained with ±5 ppm and ±0.15 min.

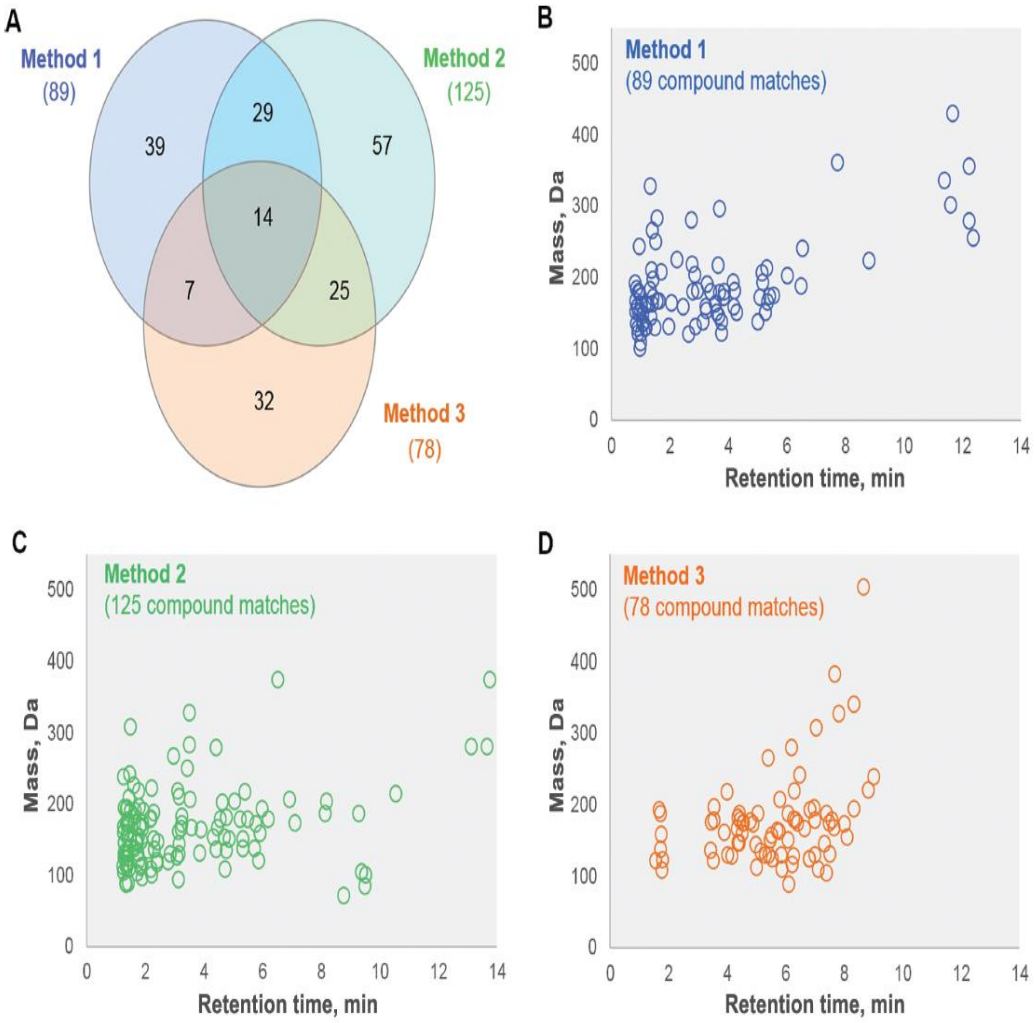


Figure 2.3. Summary of compounds identified from analysis of urine by accurate mass retention time (ARMT). (A) Venn diagram summarizing the number of AMRT matches obtained for methods 1-3. (B-D) Mass/RT scatter plots for AMRT compound matches obtained from analysis of urine by the 3 analytical methods.

2.4.3 Application of strategy to AKU: effect of nitisinone therapy on the urine metabolome

Figures 2.4 (A) and (B) show the overall study design and a representative example of AMRT compound signals extracted from the data respectively. Representative principal component analysis plots (Figure 2.4 (C)) show clear separation between the AMRT matched profiles of urine pre- vs post-nitisinone for human and mouse, showing that our strategy captured key metabolite changes.

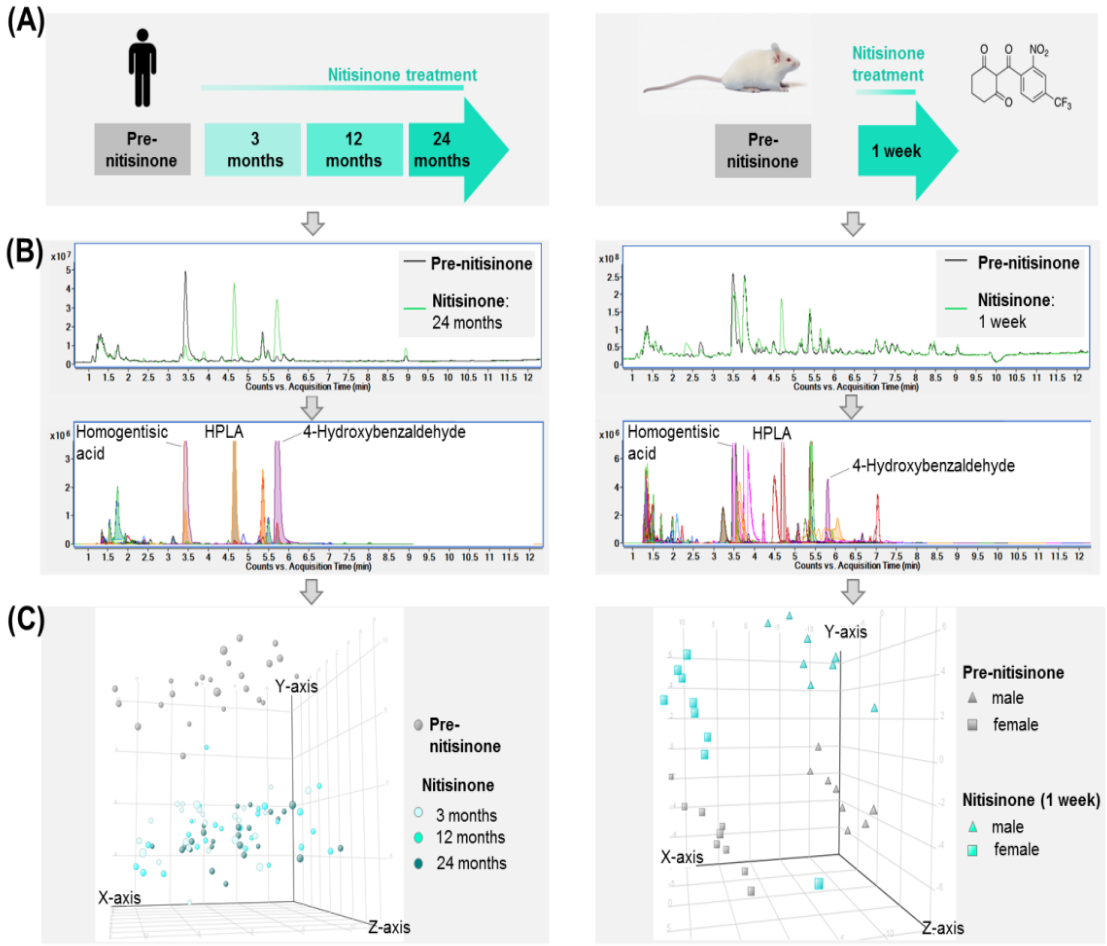


Figure 2.4. Application of the profiling strategy to AKU. (A) Urine was obtained from patients with AKU and *HGD*$^{-/-}$ mice pre- then post-nitisinone therapy. (B) Representative urine profiles (top) and extracted signals (bottom) for compounds identified by AMRT. (C) Principal component analysis

showing alteration to human (left) and mouse (right) urine metabolomes post-nitisinone. x, y and z axes represent components 1, 2 and 3, respectively.

Thirty-five metabolites showed statistically significant changes ($p<0.05$, FC>2) in abundance after 3, 12 or 24 months nitisinone in humans or 1 week on nitisinone in mice (Table 2.2). A FC >2 was chosen in order to limit false discovery and focus on clear changes. In patients with AKU, 13 metabolites increased and 14 decreased. In *HGD*$^{-/-}$ mice, 12 metabolites increased and 6 decreased. Ten metabolites changed in the same direction in humans and mice (Table 2.2). Together, the majority of these metabolite changes could be categorized into 3 main metabolic pathways: those of tyrosine, tryptophan and purine.

Compound	Fold change	Abundance		p value
		Up	Down	
3-(4-Hydroxyphenyl)lactic acid[1]	84	√		<0.0001
3-(4-Hydroxyphenyl)pyruvic acid[1]	16	√		<0.0001
3-Methoxytyramine[1]	3.3	√		<0.0001
4-Coumarate[1]	37	√		<0.0001
4-Hydroxybenzaldehyde[1]	58	√		<0.0001
4-Hydroxyphenylacetic acid [1]	2.1	√		<0.0001
L-Tyrosine[1]	100+	√		<0.0001
Mandellic acid[1]	7.0	√		<0.0001
Phenylacetic acid[1]	16.0	√		<0.0001
Tyramine[1]	9.1	√		<0.0001
Homogentisic acid[1]	5.3		√	<0.0001
Homovanillic acid[1]	4.2		√	<0.0001
3,4-Dihydroxyphenylglycol[1]	2.4		√	<0.0001
Xanthurenic acid[2]	2.4	√		<0.0001
4-Quinolinecarboxylic acid[2]	4.8	√		<0.0001
Indoxyl sulfate[2]	11.0		√	<0.0001
L-Kynurenine[2]	4.8		√	<0.0001
L-Tryptophan[2]	4.2		√	<0.0001
Caffeate[3]	2.3	√		<0.0001
3,5-Cyclic AMP[3]	4.3		√	<0.0001
3-Ureidopropionate[3]	100+		√	<0.0001
Adenine[3]	4.6		√	<0.0001
Allantoin[3]	100+		√	<0.0001
Xanthosine[3]	4.6		√	<0.0001
5-Valeroclactone[4]	6.6	√		<0.0001
Ethylmalonic acid[4]	5.2	√		<0.0001
Creatine[4]	2.1	√		<0.0001
α-ketoglutaric acid[4]	2.2	√		<0.0001
Isocitric acid[4]	3.0	√		0.002
Maleimide[4]	2.8	√		0.004
2-Hydroxybutyric acid[4]	1.7		√	<0.0001
2-Hydroxy-4-(methylthio)butyric acid[4]	100+		√	<0.0001
L-Threonine[4]	7.3		√	<0.0001
Methyl vanillate[4]	21		√	<0.0001
N-Acetylglycine[4]	2.4		√	<0.0001

Table 2.2. Urinary metabolite changes identified post-nitisinone in alkaptonuria by application of the profiling strategy. Fold changes were calculated from raw peak area. **Bold** indicates change observed in human and mouse data; *italics* indicate change observed in mouse data only; p

value <0.05 deemed significant. Metabolic pathway affected denoted by a number: 1 – tyrosine metabolism; 2- tryptophan metabolism; 3 – purine metabolism; 4 – other metabolic processes.

Interestingly, mouse data also showed clear separation by gender (principal component 1; x-axis Figure 2.4 (C)). Histamine was the primary driver of this separation in positive polarity (principal component 1 loading; 0.42), which captured the effect of gender and explained 43 % of the variation in the dataset. Histamine was significantly increased in female mice ($p<0.0001$, FC=16).

2.4.5 Alterations to non-AMRT database compounds post-nitisinone

Data showed alteration ($p<0.05$, FC>2) to metabolites with a predicted association to AKU/nitisinone metabolism that were not from AMRT databases (Supplementary Table 2.5). 2,5-Dihydroxybenzaldehyde, probably associated with ochronotic pigment, was decreased post-nitisinone. The tyrosine metabolites N-acetyl-L-tyrosine and γ-glutamyl-L-tyrosine and the nitisinone metabolite hydroxy-nitisinone were increased. These changes were observed in human and mouse urine.

2.5 Discussion

We have developed a strategy for comprehensive LC-QTOF-MS profiling with compound identification by 3 AMRT databases generated from metabolite standards. Application of this strategy enabled: (a) identification of unknown chemical entities in complex biological matrix by AMRT; and (b) identification of previously unreported changes to urinary metabolites and metabolic pathways following nitisinone treatment in AKU.

A limitation of LC-MS compound identification by public databases (Smith *et al.,* 2005; Horai *et al.,* 2010; Kanehisa *et al.,* 2010; Milman *et al.,* 2016; Wishart *et al.,* 2018) is that data were acquired using different analytical

techniques and parameters. The Metabolomics Standards Initiative (MSI) has established levels of metabolite identification confidence (Sumner *et al.,* 2007; Dunn *et al.,* 2013). Only identification strategies, as reported here, that compared 2 or more orthogonal chemical properties (*e.g.* AMRT) with an authentic standard under identical analytical conditions achieved the highest identification confidence level. Identifications by public databases in which data were acquired under different analytical conditions can only achieve the second level of confidence ('putative identification'), even with 2 matched orthogonal chemical properties.

Combining data from 3 LC techniques enhanced the number of unique urine AMRT matches obtained, improving coverage of the metabolome. Methods 1 and 2 showed some similarity in analyte retention (Figure 2.2 (B-D)). Method 2 was used because the Atlantis dC_{18} column was previously shown to chromatographically resolve metabolites of the tyrosine pathway in patients with AKU (Hughes *et al.,* 2014; Hughes *et al.,* 2015). It also provided greater overall retention of polar metabolites compared to a standard C_{18} column due to endcapping of bi-functionally bonded C_{18} stationary phase. Method 1 was included here to improve peak capacity owing to the increased theoretical plates provided by the smaller 2.1 μm column particle size. Moreover, methods 1 and 2 yielded 39 and 57 AMRT matches, respectively that were exclusive to each method (Figure 2.3 (A)).

The congenital defect that causes AKU directly affects tyrosine catabolism. However, AKU is multi-systemic (Ranganath *et al.,* 2013) and the wider metabolic consequences of the disease and nitisinone treatment have not been systematically studied. The strategy was applied to AKU, but given the range of primary and intermediary metabolism covered by the AMRT compounds, it could be applied to study metabolism in any disease. The data show that nitisinone alters tyrosine and tryptophan metabolism, and support alteration to the purine metabolic pathway. The changes observed comprise increased and decreased metabolite abundance within the same pathways, suggesting that nitisinone has complex, wide-ranging effects on metabolism. Nitisinone is licenced to treat patients with hereditary tyrosinaemia type 1

(HT1, OMIM 276700), another congenital disease of tyrosine metabolism, and is a promising HGA reducing agent in AKU. In nitisinone-treated HT1 and AKU it is established that circulating tyrosine increases markedly (Lindstedt *et al.,* 1992; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; McKiernan *et al.,* 2015; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Davison *et al.,* 2018b; Davison *et al.,* 2018c). Tyrosine is the precursor for catecholamines, thyroid hormones and melanin (Figure 2.1), suggesting that increased tyrosine substrate has potential to dysregulate these pathways. In HT1 there are concerns that hypertyrosinaemia may contribute to neurodevelopmental delay (Masurel-Paulet *et al.,* 2008; De Laet *et al.,* 2011; Thimm *et al.,* 2012; Bendadi *et al.,* 2014; McKiernan *et al.,* 2015).

Application of our profiling strategy identified a number of previously unreported metabolite changes post-nitisinone. Increased 3-(4-hydroxyphenyl)lactic acid (HPLA) is probably directly related to elevated tyrosine and/or its clearance in urine. Changes to (a) homovanillic acid and the trace amine tyramine and (b) L-tryptophan represent further alterations to dopamine (DP) and tryptophan-serotonin metabolism, respectively compared to those previously reported. L-Tryptophan is the precursor for serotonin, and a post-nitisinone decrease in the serotonin metabolite 5-hydroxyindoleacetic acid was previously reported in HT1 cerebrospinal fluid (Thimm *et al.,* 2011) and AKU urine (Davison *et al.,* 2018b), although not observed here. Xanthurenic acid and L-kynurenine were increased and decreased respectively, indicating for the first time that the kynurenine pathway, originating from tryptophan, is altered by nitisinone. Also, indoxyl sulfate, a metabolite of the indolepyruvate pathway from tryptophan, was decreased. Alterations to indolepyruvate metabolism are previously reported in nitisinone-treated AKU plasma; carboxaldehyde, indole-3-lactate and indole-3-pyruvate increased, and *in vitro* studies indicated this as a direct consequence of increased 3-(4-hydroxyphenyl)pyruvic acid (HPPA) (Lees *et al*., 1973; Gertsman *et al.,* 2015b). This is the first report of alterations to purine metabolism following treatment with nitisinone. Decreased 3,5-cyclic-AMP and xanthosine were unexpected but replicated in mice.

The remaining metabolite changes have been previously reported following nitisinone treatment; decreased HGA and increased tyrosine, N-acetyl-L-tyrosine, γ-glutamyl-tyrosine, HPPA and 3-methoxytyramine (3-MT). Decreased HGA and increased tyrosine are well-known consequences of nitisinone, and observation of these changes in each analytical run (tyrosine in positive and HGA in negative polarity) supports the analysis, data extraction workflow, and validity of the data. Increased N-acetyl-L-tyrosine and γ-glutamyl-tyrosine have been previously reported in nitisinone-treated AKU and were proportional to tyrosine elevation (Gertsman *et al.,* 2015a). Increased 3-MT was previously reported in AKU urine (Davison *et al.,* 2018b; Davison *et al.,* 2018c); it is a metabolite of DP metabolism and derived from tyrosine via decarboxylation of dihydroxyphenylalanine to DP.

Ten AMRT-matched metabolite changes were observed in human and mouse: 4-hydroxyphenylacetic acid, HPLA, HPPA, L-tyrosine, phenylacetic acid and tyramine increased post-nitisinone; 2-hydroxy-4-(methylthio)butyric acid, 3,5-cyclic AMP, HGA and xanthosine decreased post-nitisinone. The concordance between human and mouse data supports the approach and validity of the data. For mice, it was possible to control potentially confounding factors that could affect metabolism, such as diet and genetic diversity. This increases likelihood that observed changes are attributable to nitisinone. Reduced phenotypic variation could explain the prominent gender difference observed for mouse only; histamine was particularly elevated in urine from females, as previously reported in the literature for rats (Kim *et al.,* 1959; Netter *et al.,* 1961), but not mice to our knowledge.

The limitations of this study are as follows. Firstly, there is a relatively small sample size for a clinical metabolomics study (25 patients). However, AKU is a rare disease and the repeated-measures design enhanced statistical power. Secondly, data represent changes observed in urine not serum, which more closely reflects internal homeostasis. The urine metabolome is a composite of products from endogenous metabolism, diet, drugs and the gut microbiome. Urine does however give a valuable indication of the metabolic fate of the increased circulating tyrosine post-nitisinone. Further metabolomic

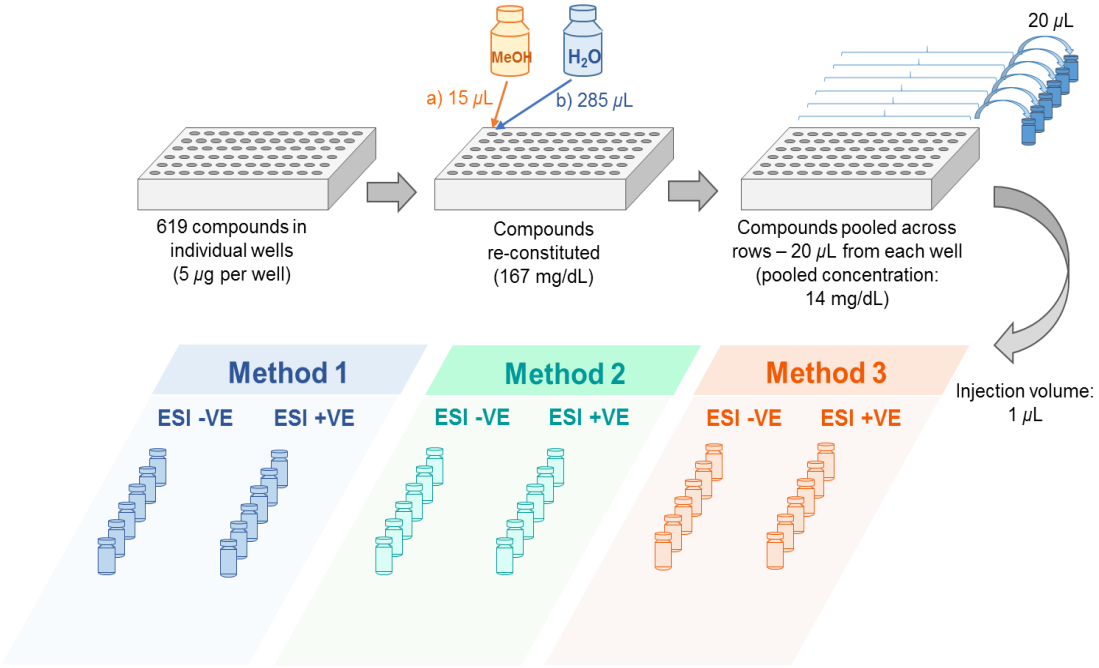
analyses on fluids and tissues from other biological locations are required to achieve more detailed, compartment-specific data, for example cerebrospinal fluid to directly study the impact of nitisinone-induced tyrosinaemia on the central nervous system. Thirdly, 3 chromatographic methods were used, however this may not always be feasible. For large-scale studies, it may be pragmatic to use 2 of the methods to reduce analytical and processing time.

2.6 Conclusion

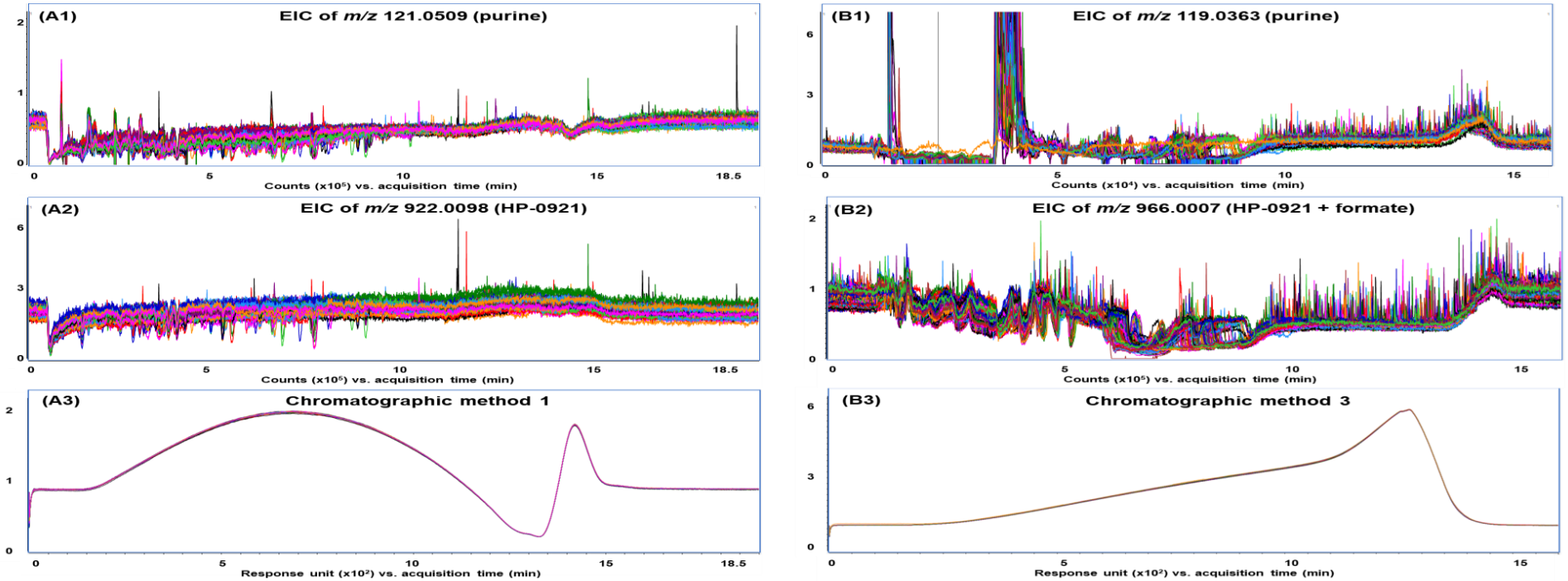
We have developed a targeted LC-QTOF-MS strategy for comprehensive coverage of the metabolome with compound structure identification using 3 AMRT databases, which are publicly available. Application of the approach to AKU has advanced our knowledge of the wider metabolic consequences of nitisinone, demonstrating the potential of our method as a metabolic phenotyping strategy more generally.

2.7 Supplementary material

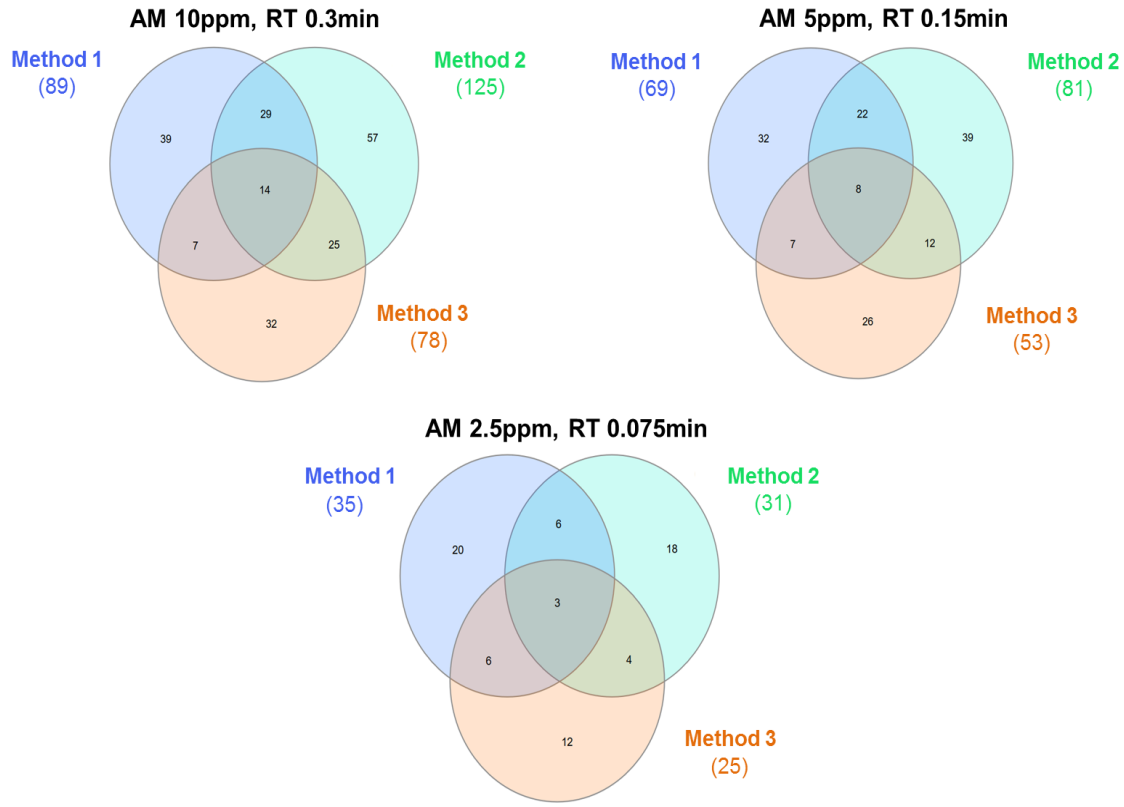
2.7.1 Supplementary Figures



Supplementary Figure 2.1. Summary of experiment workflow for preparation and LC-QTOF-MS analysis of the 619 metabolite standards. Compounds were pooled across rows (A1-A12, B1-12 *etc*) in each of the 7 96-well plates. Analysis comprised 112 injections of compound pools incorporating positive and negative ionization modes for methods 1, 2 and 3.



Supplementary Figure 2.2. Representative data from quality checks performed on data acquired from LC-QTOF-MS analysis using methods 1 and 3. Each analytical sequence consisted of 209 urine samples. The upper panels represent overlaid reference ion signal in positive polarity (A1 and A2) and negative polarity (B1 and B2), extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run. The lower panels represent overlaid binary pump pressure curves for method 1 (A3) and method 3 (B3). EIC – extracted ion chromatogram.



Supplementary Figure 2.3. Summary of the number of accurate mass retention time matches obtained by methods 1-3 with accurate mass (AM) and retention time (RT) windows of decreasing size.

2.7.2 Supplementary Tables

Plate/ Position		Compound name	Formula	Mass
1	A1	Nicotinamide adenine dinucleotide	C21H27N7O14P2	663.11
1	A2	L-Glutamine	C5H10N2O3	146.07
1	A3	Hypotaurine	C2H7NO2S	109.02
1	A4	Inosine 5'-phosphate	C10H13N4O8P	348.05
1	A5	Citrate	C6H8O7	192.03
1	A6	L-Threonine	C4H9NO3	119.06
1	A7	Purine	C5H4N4	120.04
1	A8	N-Acetylneuraminate	C11H19NO9	309.11
1	A9	L-Kynurenine	C10H12N2O3	208.08
1	A12	Pyrimidine	C4H4N2	80.04
1	A11	D-Aspartate	C4H7NO4	133.04
1	A12	Urate	C5H4N4O3	168.03
1	B1	Cytidine	C9H13N3O5	243.09
1	B2	L-Serine	C3H7NO3	105.04
1	B3	L-Cysteine	C3H7NO2S	121.02
1	B4	Citrulline	C6H13N3O3	175.1
1	B5	Taurine	C2H7NO3S	125.01
1	B6	D-Glucono-1,5-lactone	C6H12O7	178.05
1	B7	Nicotinate	C6H5NO2	123.03
1	B8	Inosine	C10H12N4O5	268.08
1	B9	4-Aminobutanoate	C4H9NO2	103.06
1	B10	Cytosine	C4H5N3O	111.04
1	B11	L-Isoleucine	C6H13NO2	131.09
1	B12	Pyrazole	C3H3N2	67.03
1	C1	L-Glutamic acid	C5H9NO4	147.05
1	C2	Ascorbate	C6H8O6	176.03
1	C3	β-Alanine	C3H7NO2	89.05
1	C4	N-Acetyl-D-glucosamine	C8H15NO6	221.09
1	C5	Glycolate	C2H4O3	76.02
1	C6	Sarcosine	C3H7NO2	89.05
1	C7	Gluconic acid	C6H12O7	196.06
1	C8	Quinate	C7H12O6	192.06
1	C9	(S)-Dihydroorotate	C5H6N2O4	158.03
1	C10	Malonate	C3H4O4	104.01
1	C11	Pipecolate	C6H11NO2	129.08
1	C12	Formamide	CH3NO	45.02
1	D1	Glycine	C2H5NO2	75.03
1	D2	L-Methionine	C5H11NO2S	149.05
1	D3	Tetrahydrofolate	C19H23N7O6	445.17

1	D4	Adenine	C5H5N5	135.05
1	D5	5'-Methylthioadenosine	C11H15N5O3S	297.09
1	D6	Thymidine	C10H14N2O5	242.09
1	D7	Glycerate	C3H6O4	106.03
1	D8	Orotate	C5H4N2O4	156.02
1	D9	Ethanolamine phosphate	C2H8NO4P	141.02
1	D10	Xanthine	C5H4N4O2	152.03
1	D11	Dihydrofolate	C19H21N7O6	443.16
1	D12	L-Cystine	C6H12N2O4S2	240.02
1	E1	L-Alanine	C3H7NO2	89.05
1	E2	L-Tryptophan	C11H12N2O2	204.09
1	E3	Uridine-5-monophosphate	C9H13N2O9P	324.04
1	E4	L-Proline	C5H9NO2	115.06
1	E5	Thymine	C5H6N2O2	126.04
1	E6	Succinate semialdehyde	C4H6O3	102.03
1	E7	(S)-Lactate	C3H6O3	90.03
1	E8	Uridine	C9H12N2O6	244.07
1	E9	Fructose-1,6-biphosphate	C6H14O12P2	340.116
1	E10	Carnosine	C9H14N4O3	226.11
1	E11	Nicotinamide	C6H6N2O	122.05
1	E12	Shikimate	C7H10O5	174.05
1	F1	Succinate	C4H6O4	118.03
1	F2	L-Phenylalanine	C9H11NO2	165.08
1	F3	Uracil	C4H4N2O2	112.03
1	F4	(S)-Malate	C4H6O5	134.02
1	F5	L-Aspartate	C4H7NO4	133.04
1	F6	2'-Deoxycytidine 5'-monophosphate	C9H14N3O7P	307.06
1	F7	Hypoxanthine	C5H4N4O	136.04
1	F8	Creatine	C4H9N3O2	131.07
1	F9	3,4-Dihydroxy-L-phenylalanine	C9H11NO4	197.07
1	F10	Guanosine	C10H13N5O5	283.09
1	F11	5,6-Dihydrouracil	C4H6N2O2	114.04
1	F12	(R)-Malate	C4H6O5	134.02
1	G1	L-Lysine	C6H14N2O2	146.11
1	G2	L-Tyrosine	C9H11NO3	181.07
1	G3	Glycerol	C3H8O3	92.05
1	G4	L-Asparagine	C4H8N2O3	132.05
1	G5	L-Valine	C5H11NO2	117.08
1	G6	Guanine	C5H5N5O	151.05
1	G7	Homoserine	C4H9NO3	119.06
1	G8	Pyridoxine	C8H11NO3	169.07
1	G9	D-AMP	C10H14N5O6P	331.07

1	G10	(R,R)-Tartaric acid	C4H6O6	150.02
1	G11	Nicotinamide mononucleotide	C11H15N2O8P	334.06
1	G12	Folic acid	C19H19N7O6	441.14
1	H1	Isocitric acid	C6H8O7	257.97
1	H2	Thiourea	CH4N2S	76.01
1	H3	Diethanolamine	C4H11NO2	105.08
1	H4	3-Aminoisobutanoate	C4H9NO2	103.06
1	H5	Cys-Gly	C5H10N2O3S	178.04
1	H6	L-2-Phosphoglyceric acid	C3H7O7P	229.96
1	H7	Guanidinoacetate	C3H7N3O2	117.05
1	H8	Creatinine	C4H7N3O	113.06
1	H9	N-Acetyl-D-tryptophan	C13H14N2O3	246.1
1	H10	Trans-aconitate	C6H6O6	174.02
1	H11	N-Acetyl-D-mannosamine	C8H15NO6	221.09
1	H12	D-Glucose-6-phosphate	C6H13O9P	282.01
2	A1	L-2,6-Diaminoheptanedioate	C7H14N2O4	190.1
2	A2	α-Aminoadipate	C6H11NO4	161.07
2	A3	Deoxycytidine	C9H13N3O4	227.09
2	A4	Noradrenaline	C8H11NO3	169.07
2	A5	D-Glucosamine 6-phosphate	C6H14NO8P	281.03
2	A6	(S,S)-Tartaric acid	C4H6O6	150.02
2	A7	3-Dehydroshikimate	C7H8O5	172.04
2	A8	Bis(3-Aminopropyl)amine	C6H17N3	131.14
2	A9	Homocysteine	C4H9NO2S	135.04
2	A12	Theophylline	C7H8N4O2	180.06
2	A11	Leucine	C6H13NO2	131.09
2	A12	D-(+)-Trehalose	C12H22O11	378.14
2	B1	Bine	C5H11NO2	117.08
2	B2	D-Tryptophan	C11H12N2O2	204.09
2	B3	3-Sulfino-L-alanine	C3H7NO4S	153.01
2	B4	O-Succinyl-L-homoserine	C8H13NO6	219.07
2	B5	Allantoin	C4H6N4O3	158.04
2	B6	Glyceraldehyde	C3H6O3	90.03
2	B7	D-Glucuronolactone	C6H10O7	176.03
2	B8	(2-Aminoethyl)phosphonate	C2H8NO3P	125.02
2	B9	Selenomethionine	C5H11NO2Se	196.106
2	B10	Maleimide	C4H3NO2	97.02
2	B11	Formate	CH2O2	46.01
2	B12	D-(+)-Glucosamine	C6H13NO5	215.06
2	C1	Paraxanthine	C7H8N4O2	180.06
2	C2	Adenosine 5'-diphosphate	C10H15N5O10P2	450.02
2	C3	2-Deoxy-D-glucose	C6H12O5	164.07
2	C4	N-(Pai)-Methyl-L-histidine	C7H11N3O2	169.09

2	C5	Galactitol	C6H14O6	182.08
2	C6	5-Oxo-D-proline	C5H7NO3	129.04
2	C7	4-Pyridoxate	C8H9NO4	183.05
2	C8	Pyridine-2,3-dicarboxylate	C7H5NO4	167.02
2	C9	Methylguanidine	C2H7N3	73.06
2	C10	Caffeine	C8H10N4O2	194.08
2	C11	DL-3-Hydroxy-3-methylglutaryl coenzyme A sodium salt hydrate	C27H42N7Na2O20P3S •3H2O	1009.67
2	C12	D-Glucuronic acid	C6H10O7	234.04
2	D1	1-Methyladenosine	C11H15N5O4	281.11
2	D2	Deoxyuridine	C9H12N2O5	228.07
2	D3	Trans-4-hydroxyproline	C5H9NO3	131.06
2	D4	Urocanate	C6H6N2O2	138.04
2	D5	Kynurenine	C10H12N2O3	208.08
2	D6	5-Oxo-L-proline	C5H7NO3	129.04
2	D7	4-Acetamidobutanoate	C6H11NO3	145.07
2	D8	Trans-Cyclohexane-1,2-diol	C6H12O2	116.08
2	D9	Melanin	C18H10N2O4	318.06
2	D10	Dopamine	C8H11NO2	189.06
2	D11	Putrescine	C4H12N2	88.1
2	D12	L-Lysine	C6H14N2O2	182.08
2	E1	Cytidine 5'-diphosphocholine	C14H26N4O11P2	532.09
2	E2	1,3-Diaminopropane	C3H10N2	74.08
2	E3	O-Phospho-L-serine	C3H8NO6P	185.01
2	E4	1-Aminocyclopropane-1-carboxylate	C4H7NO2	101.05
2	E5	5-Hydroxymethyluracil	C5H6N2O3	142.04
2	E6	L-Cystathionine	C7H14N2O4S	222.07
2	E7	L-Norvaline	C5H11NO2	117.08
2	E8	3-Hydroxy-3-methylglutarate	C6H10O5	162.05
2	E9	Phosphonoacetate	C2H5O5P	139.99
2	E10	Picolinic acid	C6H5NO2	123.03
2	E11	Ethanolamine	C2H7NO	61.05
2	E12	L-Arginine	C6H14N4O2	210.09
2	F1	4-Hydroxy-L-proline	C5H9NO3	131.06
2	F2	6-Deoxy-L-galactose	C6H12O5	164.07
2	F3	Homocystine	C8H16N2O4S2	268.06
2	F4	N-Methyl-L-glutamate	C6H11NO4	161.07
2	F5	D-Ornithine	C5H12N2O2	168.07
2	F6	Xanthosine	C10H12N4O6	284.08
2	F7	3-Methylcrotonyl-CoA	C26H42N7O17P3S	849.16
2	F8	Thyrotropin releasing hormone	C16H22N6O4	362.17
2	F9	5’-Cytidine monophosphate	C9H14N3O8P	323.05

2	F10	N-Methyl-D-aspartic acid	C5H9NO4	147.05
2	F11	Galactarate	C6H10O8	210.04
2	F12	L-Histidine	C6H9N3O2	208.05
2	G1	Nicotinic acid adenine dinucleotide phosphate	C21H27N6O18P3	766.04
2	G2	N-α-Acetyl-L-asparagine	C6H10N2O4	174.06
2	G3	L-Pipecolic acid	C6H11NO2	129.08
2	G4	D-Glucose 6-phosphate	C6H13O9P	297.99
2	G5	β-Nicotinamide adenine dinucleotide phosphate	C21H28N7O17P3	784.08
2	G6	Carbamoyl phosphate	CH4NO5P	184.95
2	G7	Isopentenyl pyrophosphate	C5H12O7P2	297.09
2	G8	Guanosine 5’-triphosphate	C10H16N5O14P3	561.98
2	G9	Thymidine-5’-diphospho-α-D-glucose	C16H26N2O16P2	610.06
2	G10	Agmatine sulfate	C5H14N4	228.09
2	G11	Glycolaldehyde dimer	C2H4O2	120.04
2	G12	2’-Deoxyguanosine 5’-triphosphate	C10H16N5O13P3	546.99
2	H1	N-Acetylglycine	C4H7NO3	117.04
2	H2	N-Acetyl-L-aspartic acid	C6H9NO5	175.05
2	H3	Inosine 5’-diphosphate	C10H14N4O11P2	493.96
2	H4	2’-Deoxyadenosine 5’-diphosphate	C10H15N5O9P2	434.02
2	H5	2’-Deoxyguanosine 5’-monophosphate	C10H14N5O7P	387.06
2	H6	Nicotinamide hypoxanthine dinucleotide	C21H26N6O15P2	688.09
2	H7	S-(5’-Adenosyl)-L-methionine	C15H22N6O5S	570.16
2	H8	6-Phosphogluconic acid	C6H13O10P	344.99
2	H9	α-Hydroxyisobutyric acid	C4H8O3	104.05
2	H10	L-Cysteic acid	C3H7NO5S	187.02
2	H11	Adenosine 5’-monophosphate	C10H14N5O7P	391.03
2	H12	D-Gluconate	C6H12O7	218.04
3	A1	Putrescine	C4H12N2	88.15
3	A2	Deoxycarnitine	C7H15NO2	181.09
3	A3	Adenosine 2’,3’-cyclic monophosphate	C10H12N5O6P	351.03
3	A4	Mevalolactone	C6H12O4	130.06
3	A5	Uridine 5’-diphosphoglucose	C15H24N2O17P2	610.02
3	A6	Isopentenyl pyrophosphate	C5H12O7P2	297.09
3	A7	2’-Deoxyuridine 5’-triphosphate	C9H15N2O14P3	489.96
3	A8	Phosphocholine	C5H14NO4P	329.03

3	A9	Uridine 5’-triphosphate	C9H15N2O15P3	484.14
3	A12	6-Hydroxydopamine	C8H11NO3	205.05
3	A11	Thiamine	C12H17N4OS	301.09
3	A12	2’-Deoxyguanosine 5’-diphosphate	C10H15N5O10P2	449.01
3	B1	5-Methylcytosine hydrochloride	C5H7N3O	161.04
3	B2	D-Glyceric acid	C3H6O4	286.02
3	B3	Cytidine 2’,3’-cyclic mono-phosphate	C9H12N3O7P	327.02
3	B4	Nε,Nε,Nε-Trimethyllysine	C9H20N2O2	223.12
3	B5	Phospho(enol)pyruvic acid	C3H5O6P	251.94
3	B6	Guanosine 5’-diphosphoglucose	C16H25N5O16P2	628.07
3	B7	α-D-Galactose 1-phosphate	C6H13O9P	425.99
3	B8	Pyridoxal 5’-phosphate	C8H10NO6P	265.04
3	B9	Dihydroxyacetone phosphate	C3H7O6P	182.01
3	B10	Phospho(enol)pyruvic acid	C3H5O6P	205.94
3	B11	D-Mannose 6-phosphate	C6H13O9P	413.93
3	B12	D-(-)-3-Phosphoglyceric acid	C3H7O7P	229.96
3	C1	L-Carnitine 2-aminoethyl dihydrogen	C7H15NO3	197.08
3	C2	Phosphate	C2H8NO4P	141.02
3	C3	O-Acetyl-L-serine	C5H9NO4	183.03
3	C4	Thymidine 5’-monophosphate	C10H15N2O8P	366.02
3	C5	Adenosine 3’,5’-cyclic monophosphate	C10H12N5O6P	369.05
3	C6	Adenosine-5’-diphosphoglucose	C16H25N5O15P2	633.05
3	C7	D-Fructose 6-phosphate	C6H13O9P	322.0
3	C8	Adenosine 3’,5’-diphosphate	C10H15N5O10P2	470.99
3	C9	3-Nitro-L-tyrosine	C9H10N2O5	226.06
3	C10	Octopamine	C8H11NO2	189.06
3	C11	N-α-Acetyl-L-lysine	C8H16N2O3	188.12
3	C12	Uridine 5’-diphosphogalactose	C15H24N2O17P2	610.02
3	D1	Spermidine	C7H19N3	253.09
3	D2	Pyridoxamine	C8H12N2O2	240.04
3	D3	5-Aminolevulinic acid	C5H9NO3	167.03
3	D4	2’-Deoxyuridine 5’-mono-phosphate	C9H13N2O8P	354.02
3	D5	Adenosine 5’-triphosphate	C10H16N5O13P3	550.96
3	D6	D-Ribulose 1,5-bisphosphate	C5H12O11P2	350.99
3	D7	Xanthosine 5’-monophosphate	C10H13N4O9P	408.01
3	D8	Flavin adenine dinucleotide	C27H33N9O15P2	847.13
3	D9	2’-Deoxyguanosine	C10H13N5O4	285.11
3	D10	Orotic acid	C5H4N2O4	174.03
3	D11	Lauroylcarnitine	C19H37NO4	343.5

3	D12	1-Methylnicotinamide	C7H9N2O	172.04
3	E1	Spermine	C10H26N4	346.12
3	E2	N-Acetyl-DL-methionine	C7H13NO3S	191.06
3	E3	Carbamoylphosphate	CH4NO5P	171.01
3	E4	5-Phospho-D-ribose 1-diphosphate	C5H13O14P3	477.88
3	E5	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate	C9H15N4O8P	338.06
3	E6	Uridine 5′-diphospho-N-acetylgalactosamine	C17H27N3O17P2	651.05
3	E7	Glyceraldehyde 3-phosphate diethyl acetal	C7H15BaO7P	379.96
3	E8	Guanosine 3’,5’-cyclic monophosphate	C10H12N5O7P	367.03
3	E9	L-Homocysteine thiolactone	C4H7NOS	153
3	E10	O-Phospho-DL-serine	C3H8NO6P	185.01
3	E11	S-(5’-Adenosyl)-L-homocysteine	C14H20N6O5S	384.12
3	E12	L-Ornithine	C5H12N2O2	168.07
3	F1	Adenine	C5H5N5	170.02
3	F2	DL-Normetanephrine	C9H13NO3	219.07
3	F3	Uridine 5’-diphospho-N-acetylglucosamine	C17H27N3O17P2	651.05
3	F4	Guanosine 5’-diphosphate	C10H15N5O11P2	466.01
3	F5	Phosphocreatine	C4H10N3O5P	257.02
3	F6	Uridine 5’-diphosphoglucuronic acid	C15H22N2O18P2	645.98
3	F7	2,3-Diphospho-D-glyceric acid	C3H8O10P2	375.87
3	F8	Cytidine 5’-diphosphate	C9H15N3O11P2	443.01
3	F9	Selenocystamine	C4H12N2Se2	283.91
3	F10	Histamine	C5H9N3	182.03
3	F11	Indoxyl sulfate	C8H7NO4S	249.96
3	F12	Ethyl 3-ureidopropionate	C6H12N2O3	160.08
3	G1	Deoxyribose	C5H10O4	134.06
3	G2	Phytic acid	C6H18O24P6	697.82
3	G3	Thiamine monophosphate	C12H17N4O4PS	416.07
3	G4	2,4-Dihydroxypyrimidine-5-carboxylic acid	C5H4N2O4	156.02
3	G5	S-Hexyl-glutathione	C16H29N3O6S	391.18
3	G6	Glyoxylic acid	C2H2O3	92.01
3	G7	Guanosine 5’-monophosphate	C10H14N5O8P	425.03
3	G8	N-Acetyl-L-alanine	C5H9NO3	131.06
3	G9	4-Guanidinobutanoate	C5H11N3O2	145.09
3	G10	Hydroxypyruvate	C3H4O4	141.97
3	G11	D-Mannosamine	C6H13NO5	215.06

3	G12	Cytochrome C	C42H52FeN8O6S2	884.28
3	H1	O-Acetyl-L-carnitine	C9H17NO4	239.09
3	H2	Riboflavin	C17H20N4O6	376.14
3	H3	Methyl β-D-galactoside	C7H14O6	194.08
3	H4	Glutaric acid	C5H8O4	132.04
3	H5	Dihydroxyfumaric acid	C4H4O6	166.01
3	H6	CMP	C9H14N3O8P	323.05
3	H7	Guanosine 5’-diphospho-D-mannose	C16H25N5O16P2	627.06
3	H8	5’-Deoxyadenosine	C10H13N5O3	251.1
3	H9	Glutathione	C10H17N3O6S	307.08
3	H10	Erythritol	C4H10O4	122.06
3	H11	Glucosaminate	C6H13NO6	195.07
3	H12	Uridine 5’-triphosphate	C9H15N2O15P3	483.97
4	A1	2’-Deoxyadenosine	C10H13N5O3	269.11
4	A2	N-Acetylputrescine	C6H14N2O	166.09
4	A3	N-Acetyl-D-galactosamine	C8H15NO6	221.09
4	A4	N-Acetyl-DL-glutamic acid	C7H11NO5	189.06
4	A5	2,4-Dihydroxypteridine	C6H4N4O2	164.03
4	A6	6-Hydroxynicotinate	C6H5NO3	139.03
4	A7	N-Acetyl-L-cysteine	C5H9NO3S	162.02
4	A8	Inosine 5’-monophosphate	C10H13N4O8P	392.01
4	A9	D-Pantothenic acid	C9H17NO5	476.17
4	A12	2-Amino-2-methylpropanoate	C4H9NO2	103.06
4	A11	Aniline-2-sulfonic acid	C6H7N	173.01
4	A12	S-Carboxymethyl-L-cysteine	C5H9NO4S	179.03
4	B1	L-Rhamnose	C6H12O5	182.08
4	B2	Thiamine pyrophosphate	C12H18N4O7P2S	460.01
4	B3	L-Histidinol	C6H11N3O	212.04
4	B4	Thymidine 5’-monophosphate	C10H15N2O8P	322.06
4	B5	3-Ureidopropionate	C4H8N2O3	132.05
4	B6	5-Aminopentanoate	C5H11NO2	117.08
4	B7	Norleucine	C6H13NO2	131.09
4	B8	N-Formylglycine	C3H5NO3	103.03
4	B9	Adenosine	C10H13N5O4	267.1
4	B10	D-(+)-Raffinose	C18H32O16	594.22
4	B11	Meso-tartaric acid	C4H6O6	168.03
4	B12	2-Acetamido-2-deoxy-β-D-glucosylamine	C8H16N2O5	220.11
4	C1	D-Saccharic acid	C6H10O8	247.99
4	C2	Adenosine 5’-triphosphate	C10H16N5O13P3	568.97
4	C3	3-Methoxy-L-tyrosine	C10H13NO4	247.11
4	C4	D-Lactose	C12H22O11	360.13

4	C5	3-Hydroxybutanoic acid	C4H8O3	104.05
4	C6	4-Imidazoleacetic acid	C5H6N2O2	161.01
4	C7	D-(+)-Galacturonic acid	C6H10O7	212.05
4	C8	Cytidine 5’-triphosphate	C9H16N3O14P3	526.95
4	C9	3’,5’-Cyclic AMP	C10H12N5O6P	329.05
4	C10	L-Methionine sulfoximine	C5H12N2O3S	180.06
4	C11	Cis-4-Hydroxy-D-proline	C5H9NO3	131.06
4	C12	N-1-Acetylspermine	C12H28N4O	352.16
4	D1	Mesoxalate	C3H2O5	197.98
4	D2	β-Nicotinamide adenine dinucleotide 2’-phosphate	C21H28N7O17P3	851.03
4	D3	3-Methylhistamine	C6H11N3	197.05
4	D4	Maleamate	C4H5NO3	115.03
4	D5	Choline	C5H14NO	104.11
4	D6	4-Aminobutanoate	C4H9NO2	139.04
4	D7	Formyl-L-methionyl peptide	C6H11NO3S	177.05
4	D8	Acetylcholine	C7H16NO2	181.09
4	D9	Oxalic Acid	C2H2O4	126.02
4	D10	5-Hydroxy-L-tryptophan	C11H12N2O3	220.08
4	D11	D-Alanine	C3H7NO2	89.05
4	D12	Theobromine	C7H8N4O2	180.06
4	E1	N-Amidino-L-aspartate	C5H9N3O4	175.06
4	E2	L-Histidine	C6H9N3O2	155.07
4	E3	L-Allothreonine	C4H9NO3	119.06
4	E4	Creatine phosphate	C4H10N3O5P	327.04
4	E5	Spermidine	C7H19N3	145.16
4	E6	Adenosine 5’-diphosphoribose	C15H23N5O14P2	581.05
4	E7	2-Methoxyethanol	C3H8O2	76.05
4	E8	Citramalate	C5H8O5	223.95
4	E9	L-Anserine	C10H16N4O3	303.12
4	E10	Biliverdin	C33H34N4O6	618.22
4	E11	Dl-5-Hydroxylysine	C6H14N2O3	198.08
4	E12	Cysteamine	C2H7NS	77.03
4	F1	Ophthalmic acid	C11H19N3O6	289.13
4	F2	L-2,3-Diaminopropionic acid	C3H8N2O2	140.04
4	F3	Trigonelline	C7H7NO2	173.02
4	F4	Adrenaline	C9H13NO3	183.09
4	F5	3,4-Dihydroxyphenyl glycol	C8H10O4	170.06
4	F6	Cadaverine	C5H14N2	174.07
4	F7	2-Hydroxybutyric acid	C4H8O3	126.03
4	F8	Coenzyme A	C21H36N7O16P3S	790.1
4	F9	Oxalomalic acid	C6H6O8	271.95
4	F10	Inosine 5’-triphosphate	C10H15N4O14P3	573.93

4	F11	Sn-Glycero-3-phosphocholine	C8H20NO6P	440.94
4	F12	2,5-Dimethylpyrazine	C6H8N2	108.07
4	G1	Stachyose	C24H42O21	666.22
4	G2	2’-Deoxycytidine 5'-diphosphate	C9H15N3O10P2	410.01
4	G3	(2r,3r)-(-)-2,3-Butanediol	C4H10O2	90.07
4	G4	D-Ribose 5-phosphate	C5H11O8P	291.99
4	G5	3-Hydroxykynurenine	C10H12N2O4	224.08
4	G6	D-(+)-Galactosamine	C6H13NO5	215.06
4	G7	2'-Deoxyadenosine 5'-triphosphate	C10H16N5O12P3	536.98
4	G8	Sn-Glycerol 3-phosphate	C3H9O6P	370.22
4	G9	Vitamin B12	C63H88CoN14O1P	1354.57
4	G10	4-Hydroxy-L-phenylglycine	C8H9NO3	167.06
4	G11	N-Acetyl-DL-serine	C5H9NO4	147.05
4	G12	Uridine 5'-diphosphate	C9H14N2O12P2	467.99
4	H1	Glycerol 2-phosphate	C3H9O6P	233.99
4	H2	α-D-Glucose 1-phosphate	C6H13O9P	322.00
4	H3	D-Glucosamine 6-sulfate	C6H13NO8S	259.04
5	A1	3-Methyglutaric acid	C6H10O4	146.06
5	A2	Sorbate	C6H8O2	150.01
5	A3	Mono-ethyl malonate	C3H4O4	132.04
5	A4	Dimethyl sulfide	C2H6S	62.02
5	A5	4-Hydroxybenzoate	C7H6O3	138.03
5	A6	Tyramine	C8H11NO	137.08
5	A7	Cortisol	C21H30O5	362.21
5	A8	Prenol	C5H10O	86.07
5	A9	3-Hydroxybenzaldehyde	C7H6O2	122.04
5	A12	Xanthurenic acid	C10H7NO4	205.04
5	A11	2-Methylpropanal oxime	C4H9NO	87.07
5	A12	Propionate	C3H6O2	96.02
5	B1	Trimethylamine	C3H9N	95.05
5	B2	Melatonin	C13H16N2O2	232.12
5	B3	Maleic acid	C4H4O4	116.01
5	B4	Pentanoate	C5H10O2	102.07
5	B5	Propanoate	C3H6O2	74.04
5	B6	Bilirubin	C33H36N4O6	584.26
5	B7	(S)-Nicotine	C10H14N2	162.12
5	B8	Butanal	C4H8O	72.06
5	B9	4-Hydroxy-2-quinolinecarboxylic acid	C10H7NO3	189.04
5	B10	2-Methylpropanoate	C4H8O2	88.05
5	B11	3-Hydroxybenzyl alcohol	C7H8O2	124.05
5	B12	Aniline	C6H7N	93.06

5	C1	4-Hydroxyphenylacetic acid	C8H8O3	152.05
5	C2	3,5-Diiodo-L-tyrosine	C9H9I2NO3	468.89
5	C3	Mandelic acid	C8H8O3	152.05
5	C4	Tryptamine	C10H12N2	160.1
5	C5	Benzoate	C7H6O2	122.04
5	C6	Glutarate	C5H8O4	132.04
5	C7	Indole-3-acetate	C10H9NO2	175.06
5	C8	Caffeate	C9H8O4	180.04
5	C9	Lumichrome	C12H10N4O2	242.08
5	C10	6-Carboxyhexanoate	C7H12O4	160.07
5	C11	N-Acetyl-L-phenylalanine	C11H13NO3	207.09
5	C12	Amylose	C14H26O11	167.04
5	D1	L-Tryptophanamide	C11H13N3O	238.07
5	D2	Phenol	C6H6O	94.04
5	D3	N-Ω-Methyltryptamine	C11H14N2	173.11
5	D4	Oxaloacetate	C4H4O5	132.01
5	D5	2,3-Dihydroxybenzoate	C7H6O4	154.03
5	D6	Propenoate	C3H4O2	72.02
5	D7	Indole-3-ethanol	C10H11NO	161.08
5	D8	Ferulate	C10H10O4	194.06
5	D9	Glycocholate	C26H43NO6	465.31
5	D10	Phenylethanolamine	C8H11NO	137.08
5	D11	Thiopurine S-methylether	C6H6N4S	166.03
5	D12	2-Hydroxy-4-(methylthio)butyric acid	C5H10O3S	338.02
5	E1	Propanal	C3H6O	58.04
5	E2	Benzoate	C7H5NaO2	144.02
5	E3	3-Amino-5-hydroxybenzoic acid	C7H7NO3	189.02
5	E4	Catechol	C6H6O2	110.04
5	E5	3,4-Dihydroxybenzoate	C7H6O4	154.03
5	E6	Cyclopentanone	C5H8O	84.06
5	E7	Pantolactone	C6H10O3	130.06
5	E8	Guaiacol	C7H8O2	124.05
5	E9	2-Hydroxyphenylacetic acid	C8H8O3	152.05
5	E10	10-Hydroxydecanoate	C10H20O3	188.14
5	E11	1,2-Didecanoyl-Sn-glycero-3-phosphocholine	C28H56NO8P	565.37
5	E12	2-Hydroxypyridine	C5H5NO	95.04
5	F1	3,4 Dihydroxyphenylacetate	C8H8O4	168.04
5	F2	N-6-(δ-2-Isopentenyl) adenine	C10H13N5	203.12
5	F3	Methyl vanillate	C8H8O4	182.06
5	F4	2-Oxobutanoate	C4H6O3	102.03
5	F5	Lipoamide	C8H15NOS2	205.06

5	F6	3-Hydroxyanthranilate	C7H7NO3	153.04
5	F7	3-(4-Hydroxyphenyl)pyruvate	C9H8O4	180.04
5	F8	Hexanoate	C6H12O2	116.08
5	F9	Methylmalonate	C4H6O4	118.03
5	F10	Serotonin creatinine sulfate complex	C14H23N5O7S	405.13
5	F11	Cortisol 21-acetate	C23H32O6	404.22
5	F12	Indole-3-acetamide	C10H10N2O	174.08
5	G1	Hippurate	C9H9NO3	179.06
5	G2	Ethylmalonic acid	C5H8O4	132.04
5	G3	3,5-Diiodo-L-thyronine	C15H13I2NO4	524.89
5	G4	Fumarate	C4H4O4	116.01
5	G5	Benzaldehyde	C7H6O	106.04
5	G6	4-Hydroxybenzaldehyde	C7H6O2	122.04
5	G7	3-(2-Hydroxyphenyl)propanoate	C9H10O3	166.06
5	G8	3-Methoxytyramine	C9H13NO2	203.07
5	G9	Benzylamine	C7H9N	107.07
5	G10	2-Quinolinecarboxylic acid	C10H7NO2	173.05
5	G11	Serotonin	C10H12N2O	211.06
5	G12	Pterin	C6H5N5O	163.05
5	H1	4-Aminobenzoate	C7H7NO2	137.05
5	H2	2-Aminophenol	C6H7NO	109.05
5	H3	Acetoin	C4H8O2	88.05
5	H4	Indole-3-pyruvic acid	C11H9NO3	203.06
5	H5	Dehydro-L-(+)-ascorbic acid dimer	C6H6O6	348.03
5	H6	3-Amino-4-hydroxybenzoic acid	C7H7NO3	153.04
5	H7	Dihydroxymandelic acid	C8H8O5	184.04
5	H8	Anthranilate	C7H7NO2	137.05
5	H9	Thioacetate	C2H4OS	76.00
5	H10	Butanoate	C4H8O2	88.05
5	H11	Indole-3-acetic acid	C10H9NO2	196.04
5	H12	5-Valerolactone	C5H8O2	100.05
6	A1	2,5-Dihydroxybenzoate	C7H6O4	154.03
6	A2	2-Methylmaleate	C5H6O4	130.03
6	A3	Hydroquinone	C6H6O2	110.04
6	A4	Dethiobiotin	C10H18N2O3	214.13
6	A5	3-Methyl-2-oxovaleric acid	C6H10O3	130.06
6	A6	α-Ketoglutaric acid	C5H6O5	167.00
6	A7	N-Acetylserotonin	C12H14N2O2	218.11
6	A8	3-Methylbutanal	C5H10O	86.07
6	A9	Itaconate	C5H6O4	130.03
6	A12	Azelaic acid	C9H16O4	188.1
6	A11	Mono-methyl glutarate	C6H10O4	146.06

6	A12	2-Methylglutaric acid	C6H10O4	146.06
6	B1	Phenylacetaldehyde	C8H8O	120.06
6	B2	2-Methylbutanal	C5H10O	86.07
6	B3	Phenyl acetate	C8H8O2	136.05
6	B4	Diacetyl	C4H6O2	86.04
6	B5	Pyruvate	C3H4O3	88.02
6	B6	Trans-Cinnamaldehyde	C9H8O	132.06
6	B7	2,6-Dihydroxypyridine	C5H5NO2	147.01
6	B8	Phenethylamine	C8H11N	121.09
6	B9	Methyl acetoacetate	C5H8O3	116.05
6	B10	Suberic acid	C8H14O4	174.09
6	B11	Adipic acid	C6H10O4	146.06
6	B12	Geranyl ayrophosphate	C10H20O7P2	368.17
6	C1	N-Acetyl-L-leucine	C8H15NO3	173.11
6	C2	2',4'-Dihydroxyacetophenone	C8H8O3	152.05
6	C3	Benzyl alcohol	C7H8O	108.06
6	C4	2-Oxoadipate	C6H8O5	160.04
6	C5	Methyl indole-3-acetate	C11H11NO2	189.08
6	C6	(R)-Mevalonic acid lithium salt	C6H11O4 · xLi+	147.15
6	C7	3-Methoxy-4-hydroxymandelate	C9H10O5	198.05
6	C8	Homovanillate	C9H10O4	182.06
6	C9	4-Quinolinecarboxylic acid	C10H7NO2	173.05
6	C10	(S)-1-Phenylethanol	C8H10O	122.07
6	C11	Tetrahydroisoquinoline	C10H13NO2	258.01
6	C12	Salicylamide	C7H7NO2	137.05
6	D1	3-Hydroxybenzoate	C7H6O3	138.03
6	D2	4-Methyl-2-oxovaleric acid	C6H10O3	130.06
6	D3	3-α,11-β,17-α,21-Tetra hydroxy-5-α-pregnan-20-one	C21H34O5	366.24
6	D4	N,N-Dimethyl-1,4-phenylenediamine	C8H12N2	136.10
6	D5	Homogentisate	C8H8O4	168.04
6	D6	Indole-3-acetaldehyde	C10H9NO	264.03
6	D7	4-Hydroxy-3-methoxyphenylglycol	C9H12O4	454.23
6	D8	3-Hydroxyphenylacetate	C8H8O3	152.05
6	D9	4-Methylcatechol	C7H8O2	124.05
6	D10	Pyridoxal	C8H9NO3	203.03
6	D11	Salicylate	C7H6O3	138.03
6	D12	3-Methylbutanol	C5H12O	88.09
6	E1	3-Methyl-2-oxindole	C9H9NO	147.07
6	E2	3-Methyladenine	C6H7N5	149.07
6	E3	3-(4-Hydroxyphenyl)lactic acid	C9H10O4	182.06

6	E4	Biotin	C10H16N2O3S	244.09
6	E5	Mercaptopyruvate	C3H4O3S	141.97
6	E6	Pyruvic aldehyde	C3H4O2	72.02
6	E7	Pyrrole-2-carboxylate	C5H5NO2	110.02
6	E8	5-Hydroxyindoleacetate	C10H9NO3	191.06
6	E9	Phenylacetic acid	C8H8O2	136.05
6	E10	Resorcinol monoacetate	C8H8O3	152.05
6	E11	Acetoacetate	C4H6O3	108.04
6	E12	Acetyl phosphate	C2H5O5P	183.95
6	F1	Ethyl 3-indoleacetate	C12H13NO2	203.09
6	F2	Dehydroascorbate	C6H6O6	174.02
6	F6	D-Fructose	C6H12O6	180.063
6	F7	L-Sorbose	C6H12O6	180.063
6	F7	Xylitol	C5H12O5	152.068
6	F9	Ribitol	C5H12O5	152.068
6	F10	Myoinositol	C6H12O6	180.063
6	F11	Mannose	C6H12O6	180.063
6	F12	Arabinose	C5H10O5	150.052
6	G1	D-Xylose	C5H10O5	150.052
6	G2	Sucrose	C12H22O11	342.116
6	G3	D-Galactose	C6H12O6	180.063
6	G4	α-D-Glucose	C6H12O6	180.063
6	G5	Allose	C6H12O6	180.063
6	G6	Mannitol	C6H14O6	182.079
6	G7	Melibiose	C12H22O11	342.116
6	G8	D-Sorbitol	C6H14O6	180.063
6	G9	Maltose	C12H22O11	342.116
6	G10	D-Tagatose	C6H12O6	180.063
6	G11	D-Gulonic acid γ-lactone	C6H12O7	178.047
6	G12	D-(-)-Arabinose	C5H10O5	150.052
6	H1	D-(+)-Cellobiose	C12H22O11	342.116
6	H2	D-Psicose	C6H12O6	180.063
6	H3	L-Arabitol	C5H12O5	152.068
6	H4	D-Lyxose	C5H10O5	150.052
6	H5	D-Ribose	C5H10O5	150.052
6	H6	Palatinose	C12H22O11	342.116
7	A1	Vitamin D2	C28H44O	396.339
7	A2	Squalene	C30H50	410.391
7	A3	4-Coumarate	C9H8O3	164.047
7	A4	Nonanoate	C9H18O2	158.13
7	A5	Chenodeoxycholate	C24H40O4	392.292
7	A6	Caprylic Acid	C8H16O2	144.115
7	A7	Cholesteryl acetate	C29H48O2	428.365

7	A8	Petroselinic acid	C18H34O2	282.255
7	A9	1,2-Dipalmitoyl-Sn-glycerol	C35H68O5	568.506
7	A12	3α,12α-Dihydroxy-5β-cholanate	C24H40O4	392.292
7	A11	3α-Hydroxy-5β-cholanate	C24H40O3	376.297
7	A12	Protoporphyrin	C34H34N4O4	562.258
7	B1	Heptanoic acid	C7H14O2	130.099
7	B2	Retinol	C20H30O	286.229
7	B3	Menaquinone	C31H40O2	444.302
7	B4	Elaidic Acid	C18H34O2	282.255
7	B5	Estradiol-17α	C18H24O2	272.177
7	B6	Myristic acid	C14H28O2	228.208
7	B7	Cholesteryl oleate	C45H78O2	650.60
7	B8	Rosmarinic acid	C18H16O8	360.084
7	B9	Glyceryl tripalmitate	C51H98O6	806.736
7	B10	Reichstein substance S	C21H30O4	346.214
7	B11	Taurolithocholate	C26H45NO5S	506.291
7	B12	Palmitoleic acid	C16H30O2	254.224
7	C1	Palmitate	C16H32O2	256.24
7	C2	3,3',5'-Triiodothyronine	C15H12I3NO4	650.79
7	C3	Sphinganine	C18H39NO2	301.298
7	C4	Lanosterol	C30H50O	426.386
7	C5	Lauric acid	C12H24O2	200.177
7	C6	Arachidic acid	C20H40O2	312.302
7	C7	Erucic acid	C22H42O2	338.318
7	C8	Deoxycholate	C24H40O4	414.274
7	C9	4-Methyl-2-oxo-pentanoic acid	C6H10O3	334.094
7	C10	Leukotriene B4	C20H32O4	336.23
7	C11	Heptadecanoate	C17H34O2	270.255
7	C12	Glyceryl trimyristate	C45H86O6	722.6424
7	D1	Linoleate	C18H32O2	280.24
7	D2	Sphingomyelin	C41H83N2O6P	730.598
7	D3	Cholesta-5,7-Dien-3β-ol	C27H44O	384.339
7	D4	Thyroxine	C15H11I4NO4	776.686
7	D5	Bis(2-Ethylhexyl)phthalate	C24H38O4	390.277
7	D6	Gamma-Linolenic acid	C18H30O2	278.224
7	D7	Ω-Hydroxydodecanoic acid	C12H24O3	216.172
7	D8	Methyl jasmonate	C13H20O3	224.141
7	D9	L-α-Phosphatidylcholine	C42H80NO8P	766.082
7	D10	Hexadecanol	C16H34O	242.26
7	D11	Dimethylbenzimidazole	C9H10N2	146.084
7	D12	Retinoate	C20H28O2	300.208
7	E1	Indole	C8H7N	117.057
7	E2	Cholate	C24H40O5	408.287

7	E3	Phylloquinone	C31H46O2	450.349
7	E4	Cholesteryl palmitate	C43H76O2	624.584
7	E5	Quinoline	C9H7N	129.057
7	E6	Docosahexaenoic acid	C22H32O2	328.24
7	E7	Diethyl 2-methyl-3-oxosuccinate	C9H14O5	202.084
7	E8	Retinyl Palmitate	C36H60O2	524.459
7	E9	1-Naphthylamine	C10H9N	143.073
7	E10	1-Hydroxy-2-naphthoate	C11H8O3	188.047
7	E11	1,2-Dipalmitoyl-rac-glycero-3-phosphoethanolamine	C37H74NO8P	691.515
7	E12	Phenylpyruvate	C9H8O3	186.029
7	F1	Trans-Cinnamate	C9H8O2	148.052
7	F2	Oleate	C18H34O2	282.255
7	F3	Stearate	C18H36O2	284.271
7	F4	β-Carotene	C40H56	536.438
7	F5	25-Hydroxycholesterol	C27H46O2	402.349
7	F6	Nervonic Acid	C24H46O2	366.349
7	F7	Desmosterol	C27H44O	384.339
7	F8	Deoxycorticosterone acetate	C23H32O4	372.23
7	F9	1-Oleoyl-rac-glycerol	C21H40O4	356.292
7	F12	α-Tocopherol	C29H50O2	430.381
7	F11	Rac-Glycerol 1-myristate	C17H34O4	302.245
7	F12	Tricosanoic acid	C23H46O2	354.349
7	G1	Coenzyme Q10	C59H90O4	863.343
7	G2	Cortisone	C21H28O5	360.193
7	G3	Decanoate	C10H20O2	172.146
7	G4	Corticosterone	C21H30O4	346.214

Supplementary Table 2.1. Compounds from the mass spectrometry metabolite library of standards. Supplied by IROA Technologies, LLC. Product information provided by Sigma-Aldrich (Version PD, MAM 09/15-1).

Compound name	Mass	RT	Polarity	
			+VE	-VE
(S)-Nicotine	162.1157	1.00	x	
1,2-Didecanoyl-Sn-glycero-3-phosphocholine	565.3744	11.98	x	x
1,2-Dipalmitoyl-Rac-glycero-3-phosphoethanolamine	691.5152	13.72	x	x
10-Hydroxydecanoate	188.1412	7.69	x	x
1-Hydroxy-2-naphthoate	188.0473	8.18	x	x
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	179.0946	0.93	x	x
1-Methyladenosine	281.1124	2.72	x	x
1-Naphthylamine	143.0735	9.30	x	
1-Oleoyl-rac-glycerol	356.2927	12.21	x	x
2,3-Dihydroxybenzoate	154.0266	3.23		x
2,4-Dihydroxyacetophenone	152.0474	5.26	x	x
2,4-Dihydroxypteridine	164.0334	1.14	x	x
2,4-Dihydroxypyrimidine-5-carboxylic acid	156.0171	0.91	x	x
2,5-Dihydroxybenzoate	154.0266	2.54		x
25-Hydroxycholesterol	402.3498	12.05	x	
2-Aminophenol	109.0528	0.97	x	
2'-Deoxyadenosine	251.1018	1.49	x	x
2'-Deoxycytidine 5'-diphosphate	387.0233	0.89		x
2'-Deoxyguanosine	267.0968	1.38	x	x
2'-Deoxyguanosine 5'-diphosphate	427.0294	0.94		x
2'-Deoxyguanosine 5'-monophosphate	347.0631	0.83	x	x
2-Hydroxy-4-(methylthio)butyric acid	150.0351	2.75	x	x
2-Hydroxybutyric acid	104.0473	1.45		x
2-Hydroxyphenylacetic acid	152.0473	4.26	x	x
2-Hydroxypyridine	95.0371	1.34	x	
2-Methylglutaric acid	146.0579	2.98	x	x
2-Methylmaleate	130.0266	1.17	x	x
2-Oxoadipate	160.0372	0.86	x	x
2-Quinolinecarboxylic acid	173.0477	3.82	x	
3-(2-Hydroxyphenyl)propanoate	166.063	5.32	x	x
3-(4-Hydroxyphenyl)lactic acid	182.0579	2.93		x
3-(4-Hydroxyphenyl)pyruvic acid	180.04	2.77		x
3,3,5-Triiodothyronine	650.79	7.94	x	x
3,4-Dihydroxybenzoate	154.0266	2.21	x	x
3,4-Dihydroxyphenyl glycol	170.0579	0.94		x
3,4-Dihydroxyphenylacetate	168.0423	2.74		x
3',5'-Cyclic AMP	329.0525	1.32	x	x
3,5-Diiodo-L-thyronine	524.8934	6.76	x	x
3,5-Diiodo-L-tyrosine	432.8672	4.45	x	x
3-Amino-4-hydroxybenzoic acid	153.0426	0.97	x	x
3-Amino-5-hydroxybenzoic acid	153.0426	0.99	x	x

3-Hydroxy-3-methylglutarate	162.0528	1.22	x	x
3-Hydroxyanthranilate	153.0426	2.49	x	x
3-Hydroxybenzaldehyde	122.0368	4.15		x
3-Hydroxybenzoate	138.0317	3.75		x
3-Hydroxybutanoic acid	104.0473	4.28	x	
3-Hydroxykynurenine	224.0797	0.91		x
3-Hydroxyphenylacetate	152.0473	3.92	x	x
3-Methoxy-4-hydroxymandelate	198.0528	1.40		x
3-Methoxy-L-tyrosine	211.0845	1.36	x	x
3-Methoxytyramine	167.0946	1.55	x	
3-Methyglutaric acid	146.0579	2.97	x	x
3-Methyl-2-Oxindole	147.0684	6.08	x	
3-Methyl-2-oxovaleric acid	130.063	2.99		x
3-Nitro-L-tyrosine	226.059	2.23	x	x
3-α,11-β,17-α,21-Tetrahydroxy-5-α-pregnan-20-one	366.2406	7.44	x	x
3-α-Hydroxy-5β-cholanate	376.2977	11.58		x
4-Acetamidobutanoate	145.0739	1.34	x	x
4-Aminobenzoate	137.0477	2.33	x	
4-Coumarate	164.0473	4.61		x
4-Hydroxy-2-quinolinecarboxylic acid	189.0426	3.30		x
4-Hydroxybenzaldehyde	122.0368	3.75	x	x
4-Hydroxybenzoate	138.0317	3.12		x
4-Hydroxyphenylacetic acid	152.0473	3.59	x	x
4-Methyl-2-Oxo-pentanoic acid	130.063	3.12		x
4-Methyl-2-Oxovaleric acid	130.063	3.23		x
4-Methylcatechol	124.0524	4.49		x
4-Pyridoxate	183.0532	1.31	x	x
4-Quinolinecarboxylic acid	173.0477	1.40	x	x
5'-Deoxyadenosine	251.1018	2.49	x	x
5-Hydroxyindoleacetate	191.0582	3.27	x	x
5-Hydroxy-L-tryptophan	220.0848	1.24		x
5'-Methylthioadenosine	297.0896	3.67	x	x
5-Oxo-D-proline	129.0426	0.90	x	x
5-Oxo-L-proline	129.0426	0.90	x	x
6-Carboxyhexanoate	160.0736	4.18	x	x
6-Hydroxydopamine	169.0739	8.08	x	x
6-Hydroxynicotinate	139.0269	1.27		x
Acetoacetate	102.0317	0.95		x
Acetoin	88.0524	9.57	x	x
Adenosine 2',3'-cyclic monophosphate	329.0525	0.93	x	x
Adenosine 3',5'-cyclic monophosphate	329.0525	1.32	x	
Adipic Acid	146.0579	2.93	x	x

Aniline	93.0578	5.61		x
Anthranilate	137.0477	4.28	x	x
Arachidic acid	312.3028	13.04		x
Azelaic acid	188.1049	6.46	x	x
Benzoic acid	122.0368	5.64		x
Biliverdin	582.2478	9.94	x	x
Biotin	244.0882	4.36	x	x
Bis(2-Ethylhexyl)phthalate	390.277	12.98	x	
Butanoate	88.0524	4.96	x	x
Caffeate	180.0423	3.84	x	x
Caffeine	194.0804	4.16	x	
Caprylic Acid	144.115	12.38	x	
Catechol	110.0368	2.75	x	x
Chenodeoxycholate	392.2927	10.98		x
Cholate	408.2876	10.34		x
Cholesta-5,7-dien-3β-ol	384.3392	12.00	x	
Cholesteryl acetate	428.3654	13.21	x	
Cholesteryl oleate	650.6002	13.37	x	
Citramalate	148.0372	1.05	x	x
Citrate	192.027	0.80	x	x
Corticosterone	346.2144	8.34		x
Cortisol	362.2093	7.72	x	x
Cortisol 21-acetate	404.2199	8.47		x
Cortisone	360.1937	7.44		x
D-AMP	331.0682	0.83	x	
Decanoate	172.1463	10.35	x	x
Deoxycholate	392.2927	11.05		x
Desmosterol	384.3392	13.16	x	
Dethiobiotin	214.1317	5.29	x	x
Dihydrofolate	443.1553	3.81	x	x
Dihydroxymandelic acid	184.0372	0.84	x	x
Dimethylbenzimidazole	146.0844	4.43	x	x
DL-Kyneurenine	208.0848	1.67	x	x
Docosahexaenoic acid	328.2402	12.18		x
Dopamine	153.079	0.80	x	x
D-Pantothenic acid	219.1107	2.73	x	x
D-Tryptophan	204.0899	2.82	x	x
Elaidic acid	282.2559	12.51		x
Erucic acid	338.3185	13.10		x
Ethyl 3-indoleacetate	203.0946	7.91	x	
Ethylmalonic acid	132.0423	1.95		x
Ferulate	194.0579	5.15	x	x
Flavin adenine dinucleotide	785.1571	3.92	x	x

Folic acid	441.1397	3.89	x	x
Formyl-L-methionyl peptide	177.046	2.71	x	x
Fumarate	116.011	0.87		x
γ–Linoleic acid	278.2246	11.95	x	
Glyceryl trimyristate	722.6424	13.32	x	
Glycocholate	465.309	9.68	x	x
Guanosine	283.0917	1.19	x	x
Guanosine 3',5'-cyclic monophosphate	345.0474	1.14	x	x
Guanosine 5'-monophosphate	363.058	0.89	x	x
Heptadecanoate	270.2559	12.56		x
Heptanoic acid	130.0994	11.10	x	
Hippurate	179.0582	3.67	x	x
Homogentisate	168.0423	1.62	x	x
Homovanillate	182.0579	4.19		x
Hydroquinone	110.0368	2.54		x
Hypoxanthine	136.0385	0.85	x	x
Indole-3-acetaldehyde	159.0684	2.43	x	x
Indole-3-acetamide	174.0793	4.21	x	x
Indole-3-acetate	175.0633	5.53	x	x
Indole-3-acetic acid	175.0633	5.53	x	x
Indole-3-ethanol	161.0841	5.60	x	
Indoxyl sulfate	213.0096	2.48		x
Inosine	268.0808	1.19	x	x
Itaconate	130.0266	1.77	x	x
Lanosterol	426.3862	13.46	x	
Lauric acid	200.1776	11.25	x	x
Lauroylcarnitine	343.2723	9.61	x	x
Leucine	131.0946	1.26	x	x
Leukotriene B4	336.2301	11.38	x	x
Linoleate	280.2402	12.21	x	x
Lipoamide	205.0595	6.40	x	
L-Isoleucine	131.0946	1.17	x	x
L-Kynurenine	208.0848	1.67	x	x
L-Methionine	149.051	0.84	x	x
L-Phenylalanine	165.079	2.05	x	x
L-Tryptophan	204.0899	2.82	x	x
L-Tryptophanamide	203.1059	2.44	x	x
L-Tyrosine	181.0739	0.92	x	x
Lumichrome	242.0804	6.51	x	x
L-α-Phosphatidylcholine	757.5622	13.55	x	x
Mandelic Acid	152.0473	3.10		x
Melatonin	232.1212	5.60	x	x
Menaquinone	444.3028	6.16		x

Mercaptopyruvate	119.9881	1.99		x
Meso-tartaric acid	150.0164	0.91		x
Methyl indole-3-acetate	189.079	6.97	x	
Methyl jasmonate	224.1412	8.79	x	
Methyl vanillate	168.0423	4.74		x
Methylmalonate	118.0266	1.04		x
Mevalolactone	148.0736	1.20	x	x
Mono-methyl glutarate	146.0579	3.64	x	x
Myristic acid	228.2089	11.89	x	
N,N-Dimethyl-1,4-phenylenediamine	136.10	1.04	x	
N-6-(δ-2-Isopentenyl)-adenine	203.1171	5.99	x	x
N-Acetyl-DL-glutamic acid	189.0637	1.27	x	x
N-Acetyl-DL-methionine	191.0616	3.26	x	x
N-Acetyl-D-tryptophan	246.1004	5.19	x	x
N-Acetyl-L-alanine	131.0582	1.13	x	x
N-Acetyl-L-cysteine	163.0303	1.37	x	x
N-Acetyl-L-leucine	173.1052	5.06	x	x
N-Acetyl-L-phenylalanine	207.0895	5.14	x	x
N-Acetylserotonin	218.1055	3.64	x	x
Nervonic Acid	366.3498	13.44		x
Nicotinamide	122.048	1.00	x	
Nicotinate	123.032	0.89	x	x
Nonanoate	158.1307	10.75	x	x
Norleucine	131.0946	1.33	x	x
Oleate	282.2559	12.47		x
Palmitate	256.2402	12.36	x	x
Palmitoleic acid	254.2246	12.04		x
Paraxanthine	180.0647	3.24	x	
Petroselinic acid	282.2559	12.5		x
Phenethylamine	121.0891	2.62	x	
Phenyl acetate	136.0524	3.97		x
Phenylacetic acid	136.0524	2.93		x
Phenylethanolamine	137.0841	1.55	x	
Phenylpyruvate	164.0473	3.55		x
Phylloquinone	450.3498	11.72	x	
Propenoate	72.0211	4.88		x
Protoporphyrin	562.258	12.41		x
Pterin	163.0494	1.10	x	x
Purine	120.0436	1.09	x	x
Pyridine-2,3-dicarboxylate	167.0219	0.83	x	x
Pyridoxal	167.0582	0.86		x
Pyridoxal 5'-phosphate	247.0246	1.08	x	x
Pyridoxine	169.0739	0.86	x	x

Pyrrole-2-carboxylate	111.032	2.79		x
Pyruvic aldehyde	72.0211	2.20		x
Quinoline	129.0578	9.00	x	
Rac-glycerol 1-myristate	302.2457	11.57	x	x
Reichstein's substance S	346.2144	8.43		x
Resorcinol monoacetate	152.0473	5.25	x	x
Retinoate	300.2089	12.10		x
Riboflavin	376.1383	4.88		x
Rosmarinic acid	360.0845	5.81		x
S-(5'-Adenosyl)-L-homocysteine	384.1216	1.01	x	x
Salicylamide	137.0477	4.10	x	x
Salicylate	138.0317	4.99	x	x
Selenomethionine	191.0015	0.97		x
Serotonin	176.095	1.27	x	
S-Hexyl-glutathione	391.1777	7.11	x	x
Sphinganine	301.2981	11.03	x	x
Sphingomyelin	730.5989	13.65	x	x
Stearate	284.2715	12.73		x
Suberic acid	174.0892	5.39	x	x
Succinic acid	118.0266	1.01		x
Taurolithocholate	483.3018	10.32		x
Theobromine	180.0647	2.75	x	
Theophylline	180.0647	3.37	x	x
Thiopurine S-methylether	166.0313	4.19	x	x
Thymidine	242.0903	2.17	x	x
Thymidine 5'-monophosphate	322.0566	0.93	x	x
Thymine	126.0429	1.26		x
Thyrotropin releasing hormone	362.1703	1.02	x	x
Thyroxine	776.6867	8.70	x	x
Trans-aconitate	174.0164	0.98	x	x
Trans-cinnamaldehyde	148.0524	6.97		x
Trans-cinnamate	148.0524	6.94	x	x
Trans-cyclohexane-1,2-diol	116.0837	3.29	x	
Tricosanoic acid	354.3498	13.59		x
Tryptamine	160.10	3.20	x	
Tyramine	137.0841	1.07	x	
Uridine	244.0695	0.93	x	x
Xanthine	152.0334	0.90	x	x
Xanthosine	284.0757	1.55	x	x
Xanthurenic acid	205.0375	2.91	x	x
α-Hydroxyisobutyric acid	104.0473	1.41		x
α-Tocopherol	430.3811	11.65	x	
β-Carotene	536.4382	13.93	x	x

Ω-Hydroxydodecanoic acid	216.1725	9.02	x	x

Supplementary Table 2.2. Method 1 accurate mass retention time data from the analysis of mass spectrometry metabolite library of standards. Supplied by IROA Technologies, LLC. Product information provided by Sigma-Aldrich (Version PD, MAM 09/15-1). RT – retention time; +VE – positive; -VE – negative; x – detected in respective polarity.

Compound name	Mass	RT	Polarity	
			+VE	-VE
(2-Aminoethyl)phosphonate	125.02	1.25	x	x
(R)-Malate	134.02	1.58	x	x
(R,R)-Tartaric acid	150.02	1.46		x
(S)-Dihydroorotate	158.03	1.61	x	x
(S)-Malate	134.02	1.58	x	x
(S,S)-Tartaric acid	150.02	1.46		x
1,2-Didecanoyl-Sn-glycero-3-phosphocholine	565.37	13.32	x	x
10-Hydroxydecanoate	188.14	9.27	x	x
1-Aminocyclopropane-1-carboxylate	101.05	1.30	x	
1-Hydroxy-2-Naphthoate	188.05	9.90	x	
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	179.09	2.15	x	x
1-Methyladenosine	281.11	4.39	x	x
1-Naphthylamine	143.07	10.71	x	
1-Oleoyl-rac-glycerol	356.29	14.02		x
2,3-Dihydroxybenzoate	154.03	6.26		x
2,4-Dihydroxyacetophenone	152.05	3.22	x	x
2,4-Dihydroxypteridine	164.03	2.86	x	x
2,4-Dihydroxypyrimidine-5-carboxylic acid	156.02	2.22	x	
2,5-Dihydroxybenzoate	154.03	4.67		x
2,5-Dimethylpyrazine	108.07	9.74	x	
2-Amino-2-methylpropanoate	103.06	1.35	x	
2-Aminophenol	109.05	2.16	x	
2-Deoxyadenosine	251.1	3.40	x	
2-Deoxycytidine 5-monophosphate	307.06	1.57		x
2-Deoxy-D-Glucose	164.07	1.43	x	x
2-Deoxyguanosine	267.1	3.22	x	
2-Deoxyguanosine 5-monophosphate	347.06	2.21	x	x
2-Deoxyuridine 5-mono-phosphate	308.04	2.04	x	x
2-Hydroxy-4-(methylthio)butyric acid	150.04	4.92	x	x
2-Hydroxybutyric acid	104.05	3.13		x
2-Hydroxyphenylacetic acid	152.05	6.00	x	x
2-Hydroxypyridine	95.037	3.11	x	
2-Methylglutaric acid	146.06	4.74	x	x
2-Methylmaleate	130.03	2.84	x	x
2-Oxoadipate	160.04	2.08		x
2-Quinolinecarboxylic acid	173.05	5.75	x	x
3-(2-Hydroxyphenyl)propanoate	166.06	7.09		x
3-(4-Hydroxyphenyl)lactic acid	182.06	4.75		x
3-(4-Hydroxyphenyl)pyruvic acid	180.04	5.69		x

3,3,5-Triiodothyronine	650.79	9.38	x	
3,4-Dihydroxybenzoate	154.03	4.31	x	x
3,4-Dihydroxy-L-phenylalanine	197.07	1.77	x	
3,4-Dihydroxyphenyl glycol	170.06	2.23		x
3,4-Dihydroxyphenylacetate	168.04	4.43	x	x
3,5-Cyclic AMP	329.05	3.47	x	x
3,5-Diiodo-L-thyronine	524.89	8.32	x	x
3,5-Diiodo-L-tyrosine	432.87	6.17	x	x
3-Amino-4-hydroxybenzoic acid	153.04	2.43	x	x
3-Amino-5-hydroxybenzoic acid	153.04	2.43	x	x
3-Aminoisobutanoate	103.06	1.29	x	
3-Dehydroshikimate	172.04	1.82		x
3-Hydroxy-3-methylglutarate	162.05	2.89	x	x
3-Hydroxyanthranilate	153.04	4.62	x	x
3-Hydroxybenzaldehyde	122.04	6.10		x
3-Hydroxybenzoate	138.03	5.70	x	x
3-Hydroxybenzyl Alcohol	124.05	4.70		x
3-Hydroxybutanoic acid	104.05	5.77	x	x
3-Hydroxykynurenine	224.08	2.19	x	x
3-Hydroxyphenylacetate	152.05	5.69	x	x
3-Methoxy-4-hydroxymandelate	198.05	3.22	x	x
3-Methoxy-L-tyrosine	211.08	3.13	x	x
3-Methoxytyramine	167.09	3.20	x	
3-Methyglutaric acid	146.06	4.71	x	x
3-Methyl-2-oxindole	147.07	7.84	x	
3-Methyl-2-oxovaleric acid	130.06	5.09		x
3-Methyladenine	149.07	1.70	x	
3-Methylhistamine	125.1	1.26	x	
3-Nitro-L-tyrosine	226.06	4.06	x	x
3-Sulfino-L-alanine	153.01	1.39	x	
3-Ureidopropionate	132.05	1.72	x	x
3-α,11-β,17-α,21-Tetrahydroxy- 5-α-pregnan-20-one	366.24	9.17	x	x
3α,12α-Dihydroxy-5β-cholanate	392.29	13.74	x	x
3α-Hydroxy-5β-cholanate	376.3	13.73	x	x
4-Acetamidobutanoate	145.07	3.14	x	x
4-Aminobenzoate	137.05	4.41	x	x
4-Coumarate	164.05	6.68	x	
4-Guanidinobutanoate	145.09	1.49		x
4-Hydroxy-2-quinolinecarboxylic acid	189.04	5.31	x	x
4-Hydroxy-3-methoxyphenylglycol	184.07	3.73	x	x
4-Hydroxybenzaldehyde	122.04	5.84	x	x
4-Hydroxybenzoate	138.03	5.31		x

4-Hydroxy-L-phenylglycine	167.06	1.37	x	
4-Hydroxy-L-proline	131.06	1.46	x	x
4-Hydroxyphenylacetic acid	152.05	5.31	x	x
4-Imidazoleacetic acid	126.04	1.29	x	x
4-Methyl-2-oxo-pentanoic acid	130.06	5.15	x	x
4-Methyl-2-oxovaleric acid	130.06	5.36		x
4-Methylcatechol	124.05	6.41		x
4-Pyridoxate	183.05	3.22	x	x
4-Quinolinecarboxylic acid	173.05	3.23	x	x
5,6-Dihydrouracil	114.04	1.32	x	
5-Aminoimidazole-4-carboxamide-1-B-D-ribofuranosyl 5-monophosphate	338.06	1.71		x
5-Aminopentanoate	117.08	5.33	x	
5-CMP	323.05	1.59	x	x
5-Deoxyadenosine	251.1	4.13	x	x
5-Hydroxyindoleacetate	191.06	5.02	x	x
5-Hydroxy-L-tryptophan	220.08	3.08	x	x
5-Hydroxymethyluracil	142.04	1.74		x
5-Methylcytosine hydrocloride	125.06	1.39	x	
5-Methylthioadenosine	297.09	5.34	x	x
5-Oxo-D-proline	129.04	2.13	x	x
5-Oxo-L-proline	129.04	2.12	x	x
5-Valerolactone	100.05	7.14		x
6-Carboxyhexanoate	160.07	5.90	x	x
6-Deoxy-L-galactose	164.07	1.57	x	x
6-Hydroxynicotinate	139.03	3.24	x	x
Acetoacetate	102.03	2.19		x
Acetoin	88.052	1.34	x	
Adenine	135.05	1.64	x	x
Adenine hydrochloride hydrate	135.05	1.69	x	x
Adenosine	267.1	3.22	x	x
Adenosine 2,3-cyclic monophosphate	329.05	2.70	x	x
Adenosine 3,5-cyclic monophosphate	329.05	3.47	x	x
Adenosine 3,5-diphosphate	427.03	1.64	x	
Adenosine 5-diphosphoribose	559.07	1.65	x	
Adenosine 5-monophosphate	347.06	1.82	x	x
Adenosine-5-diphosphoglucose	589.08	1.65	x	x
Adipic acid	146.06	4.62	x	x
Allantoin	158.04	1.36		x
Allose	180.06	1.35	x	x
Amylose	370.15	10.28	x	
Aniline	93.058	7.33		x
Anthranilate	137.05	6.21		x

Arabinose	150.05	1.38		x
Arachidic acid	312.3	14.30	x	x
Ascorbate	176.03	1.61	x	
Azelaic acid	188.1	8.12	x	x
Benzaldehyde	106.04	9.37	x	
Benzoic acid	122.04	7.54		x
Benzylamine	107.07	3.05	x	
Biliverdin	582.25	11.34	x	
Biotin	244.09	5.98		x
Bis(2-ethylhexyl)phthalate	390.28	13.51	x	x
Bis(3-aminopropyl)amine	131.14	1.41	x	
Butanal	72.058	8.76	x	
Caffeate	180.04	6.17	x	x
Caffeine	194.08	5.97	x	
Catechol	110.04	4.70	x	
Chenodeoxycholate	392.29	12.20	x	x
Cholate	408.29	11.61	x	x
Cholesteryl acetate	428.37	12.49	x	
Cis-4-hydroxy-D-proline	131.06	1.29	x	x
Citramalate	148.04	2.41	x	x
Citrate	192.03	1.92		x
Citrulline	175.1	1.29	x	x
Corticosterone	346.21	9.90	x	
Cortisol	362.21	9.27	x	x
Cortisol 21-acetate	404.22	9.98	x	x
Creatine	131.07	1.32	x	
Creatine phosphate	211.04	1.42	x	x
Creatinine	113.06	1.21	x	x
Cyclopentanone	84.058	8.16	x	
Cytidine	243.09	1.45	x	x
Cytidine 2,3-cyclic monophosphate	305.04	1.48	x	x
Cytidine 5-diphosphocholine	488.11	1.32	x	x
Cytosine	111.04	1.44	x	
D-(-)-3-Phosphoglyceric acid	185.99	1.57	x	
D-(-)-Arabinose	150.05	1.37		x
D-(+)-Cellobiose	342.12	1.39	x	x
D-(+)-Galacturonic acid	194.04	1.34		x
D-(+)-Raffinose	504.17	1.45	x	x
D-(+)-Trehalose	342.12	1.37	x	x
D-Alanine	89.048	1.42	x	
D-AMP	331.07	2.19	x	x
D-Aspartate	133.04	1.28	x	x
Dehydroascorbate	174.02	1.51		x

Dehydro-L-(+)-ascorbic acid dimer	174.02	1.37	x	x
Deoxycarnitine	145.11	1.30	x	
Deoxycholate	392.29	12.29	x	
Deoxycorticosterone acetate	372.23	11.26	x	
Deoxycytidine	227.09	1.58	x	x
Deoxyribose	134.06	8.91	x	
Dethiobiotin	214.13	6.93		x
D-Fructose 6-phosphate	260.03	1.40	x	x
D-Galactose	180.06	1.33	x	x
D-Gluconate	196.06	1.36	x	x
D-Glucono-1,5-lactone	196.06	1.36	x	x
D-Glucosamine 6-phosphate	259.05	1.22	x	x
D-Glucose 6-phosphate	260.03	1.39	x	x
D-Glucuronic acid	194.04	1.33	x	x
D-Glucuronolactone	194.04	1.32	x	x
D-Glyceric acid	106.03	1.46		x
D-Gulonic acid g-lactone	196.06	1.35		x
Diacetyl	86.037	9.48	x	
Dihydrofolate	443.16	5.33	x	x
Dihydroxyacetone phosphate	170	1.43	x	x
Dihydroxymandelic acid	184.04	1.94	x	x
Dimethylbenzimidazole	146.08	6.05	x	
D-Lactose	342.12	1.33	x	x
DL-Kyneurenine	208.08	3.51	x	x
DL-Normetanephrine	183.09	1.61	x	
D-Lyxose	150.05	1.37	x	x
D-Mannosamine	179.08	1.27	x	x
D-Mannose 6-phosphate	260.03	1.32	x	x
Docosahexaenoic acid	328.24	13.29	x	x
Dopamine	153.08	1.66	x	x
D-Pantothenic acid	219.11	4.34	x	x
D-Psicose	180.06	1.40	x	x
D-Ribose	150.05	1.41	x	x
D-Ribose 5-phosphate	230.02	1.41	x	
D-Saccharic acid	210.04	1.42	x	
D-Sorbitol	182.08	1.35	x	x
D-Tagatose	180.06	1.36	x	x
D-Tryptophan	204.09	4.60	x	x
D-Xylose	150.05	1.37	x	x
Elaidic acid	282.26	13.64	x	x
Adrenaline	183.09	1.38	x	x
Erucic acid	338.32	14.35		x
Erythritol	122.06	1.37	x	x

Estradiol-17α	272.18	9.97		x
Ethyl 3-indoleacetate	203.09	9.50	x	
Ethylmalonic acid	132.04	3.83	x	x
Ferulate	194.06	7.04		x
Flavin adenine dinucleotide	785.16	5.97	x	x
Folic acid	441.14	5.43	x	x
Formyl-L-methionyl peptide	177.05	4.55	x	
Fumarate	116.01	2.12		x
Galactarate	210.04	1.35		x
Galactitol	182.08	1.32	x	x
γ–Linoleic acid	278.22	13.51	x	x
Gluconic acid	196.06	1.37	x	
Glucosaminate	195.07	1.25	x	x
Glutathione	307.08	1.80		x
Glyceraldehyde	90.032	1.40	x	
Glycerate	106.03	2.27		x
Glycerol	92.047	1.42	x	
Glycerol 2-phosphate	172.01	1.45	x	x
Glyceryl trimyristate	722.64	14.64	x	
Glycine	75.032	1.23	x	
Glycocholate	465.31	11.04	x	x
Glyoxylic acid	74.0	1.49		x
Guaiacol	124.05	10.49	x	
Guanidinoacetate	117.05	1.39	x	
Guanine	151.05	1.72	x	
Guanosine	283.09	2.98		x
Guanosine 3,5-cyclic monophosphate	345.05	3.46	x	x
Guanosine 5-diphospho-D-mannose	605.08	1.75	x	x
Guanosine 5-diphosphoglucose	605.08	1.83	x	x
Guanosine 5-monophosphate	363.06	1.88	x	x
Heptadecanoate	270.26	13.71		x
Hexadecanol	242.26	12.6	x	
Hippurate	179.06	5.48	x	x
Homocysteine	135.04	1.41	x	
Homocystine	268.06	1.42	x	x
Homogentisate	168.04	3.54		x
Homoserine	119.06	1.27	x	x
Homovanillate	182.06	5.89		x
Hydroquinone	110.04	4.67		x
Hydroxypyruvate	104.01	1.44	x	
Hypotaurine	109.02	1.28	x	x
Hypoxanthine	136.04	2.07	x	x
Indole-3-acetaldehyde	159.07	4.66	x	x

Indole-3-acetamide	174.08	6.04	x	x
Indole-3-acetate	175.06	7.27		x
Indole-3-acetic acid	175.06	7.27		x
Indole-3-ethanol	161.08	5.94		x
Indole-3-pyruvic acid	203.06	6.00	x	
Indoxyl sulfate	213.01	5.03		x
Inosine	268.08	2.96	x	x
Inosine 5-monophosphate	348.05	1.95	x	x
Inosine 5-phosphate	348.05	1.93	x	x
Isocitric acid	192.03	1.63	x	x
Itaconate	130.03	3.44	x	x
L-Alanine	89.048	1.26	x	
L-Allothreonine	119.06	1.29	x	x
Lanosterol	426.39	14.08		x
L-Arabitol	152.07	1.36	x	x
L-Asparagine	132.05	1.25	x	x
L-Aspartate	133.04	1.28	x	x
Lauric acid	200.18	12.52	x	x
Lauroylcarnitine	343.27	10.93	x	x
L-Carnitine	161.11	1.23	x	x
L-Cystathionine	222.07	1.21	x	x
L-Cysteic acid	169	1.38	x	x
L-Cysteine	121.02	1.35	x	
L-Cystine	240.02	1.23	x	x
Leucine	131.09	2.74	x	x
L-Glutamic acid	147.05	1.30	x	
L-Glutamine	146.07	1.26	x	x
L-Homocysteine thiolactone	117.02	1.34	x	
Linoleate	280.24	13.38		x
Lipoamide	205.06	8.16	x	
L-Isoleucine	131.09	2.55	x	x
L-Kynurenine	208.08	3.50	x	x
L-Methionine	149.05	1.85	x	x
L-Methionine sulfoximine	180.06	1.25	x	x
L-Norvaline	117.08	1.71	x	x
L-Phenylalanine	165.08	3.88	x	x
L-Pipecolic acid	129.08	1.68	x	
L-Proline	115.06	1.40	x	
L-Rhamnose	164.07	1.47		x
L-Serine	105.04	1.25	x	x
L-Sorbose	180.06	1.35	x	x
L-Threonine	119.06	1.29	x	x
L-Tryptophan	204.09	4.60	x	x

L-Tryptophanamide	203.11	4.05	x	x
L-Tyrosine	181.07	2.17	x	x
Lumichrome	242.08	8.31	x	
L-Valine	117.08	1.65	x	x
Maleamate	115.03	1.87	x	
Maleic Acid	116.01	2.01		x
Maleimide	97.016	1.87	x	
Malonate	104.01	1.71		x
Maltose	342.12	1.38	x	x
Mandelic acid	152.05	4.85	x	x
Mannitol	182.08	1.35	x	x
Mannose	180.06	1.35	x	x
Melatonin	232.12	7.33	x	x
Melibiose	342.12	1.34	x	x
Mercaptopyruvate	119.99	1.99		x
Meso-tartaric acid	150.02	1.44		x
Methyl indole-3-acetate	189.08	7.25	x	
Methyl jasmonate	224.14	10.21	x	x
Methyl vanillate	168.04	6.54		x
Methyl β-D-galactoside	194.08	1.56	x	x
Methylguanidine	73.064	1.25	x	
Methylmalonate	118.03	1.86	x	
Mevalolactone	148.07	2.69		x
Mono-ethyl malonate	104.01	1.61	x	
Mono-methyl glutarate	146.06	5.29	x	x
Myoinositol	180.06	1.29	x	x
Myristic acid	228.21	13.08		x
N,N-Dimethyl-1,4-phenylenediamine	136.1	2.31	x	
N-6-(δ2-isopentenyl)-adenine	203.12	7.91		x
N-Acetyl-D-galactosamine	221.09	1.44	x	
N-Acetyl-D-glucosamine	221.09	1.44	x	x
N-Acetyl-DL-glutamic acid	189.06	2.26	x	x
N-Acetyl-DL-methionine	191.06	5.05	x	x
N-Acetyl-DL-serine	147.05	1.67	x	x
N-Acetyl-D-mannosamine	221.09	1.39	x	x
N-Acetyl-D-tryptophan	246.1	7.02	x	x
N-Acetylglycine	117.04	1.78		x
N-Acetyl-L-alanine	131.06	2.58	x	x
N-Acetyl-L-aspartic acid	175.05	1.89	x	x
N-Acetyl-L-cysteine	163.03	3.11	x	x
N-Acetyl-L-leucine	173.11	6.75		x
N-Acetyl-L-phenylalanine	207.09	6.90		x
N-Acetylneuraminate	309.11	1.47	x	x

N-Acetylputrescine	130.11	1.37	x	
N-Acetylserotonin	218.11	5.38	x	x
Nicotinamice adeninine dinucleotide	663.11	1.76	x	x
N-Amidino-L-aspartate	175.06	1.33	x	x
N-Formylglycine	103.03	1.56	x	x
Nicotinamide	122.05	2.39	x	
Nicotinamide hypoxanthine dinucleotide	664.09	1.91	x	x
Nicotinamide mononucleotide	334.06	1.40	x	
Nicotinate	123.03	2.11	x	x
N-Methyl-D-aspartic acid	147.05	1.32	x	
N-Methyl-L-glutamate	161.07	1.35	x	x
Nonanoate	158.13	12.28	x	
Noradrenaline	169.07	1.23	x	x
Norleucine	131.09	2.78	x	x
N-α-Acetyl-L-asparagine	174.06	1.61	x	x
N-α-Acetyl-L-lysine	188.12	1.37		x
O-Acetyl-L-serine	147.05	1.43	x	
Octopamine	153.08	1.39	x	
Oleate	282.26	13.10	x	x
O-Phospho-L-serine	185.01	1.36	x	
O-Phosphorylethanolamine	141.02	1.25	x	x
Orotate	156.02	1.75		x
Orotic acid	156.02	1.76	x	x
O-Succinyl-L-homoserine	219.07	1.76	x	x
Palatinose	342.12	1.40	x	x
Palmitate	256.24	13.52	x	x
Palmitoleic acid	254.22	13.2		x
Paraxanthine	180.06	5.03	x	
Pentanoate	102.07	9.49	x	
Petroselinic acid	282.26	13.11	x	x
Phenethylamine	121.09	4.16	x	
Phenol	94.042	9.82	x	
Phenyl acetate	136.05	3.97		x
Phenylacetaldehyde	120.06	9.94	x	
Phenylacetic acid	136.05	4.75		x
Phenylethanolamine	137.08	3.02	x	
Phospho(enol)pyruvic acid	167.98	1.47	x	x
Phosphocholine	183.07	1.30	x	x
Phosphonoacetate	139.99	1.60		x
Phylloquinone	450.35	12.56	x	
Picolinic acid	123.03	1.79	x	
Pipecolate	129.08	1.69	x	
Propenoate	72.021	6.52		x

Protoporphyrin	562.26	13.69	x	x
Pterin	163.05	2.83	x	x
Purine	120.04	2.75	x	
Pyridine-2,3-dicarboxylate	167.02	2.12	x	x
Pyridoxal	167.06	1.84		x
Pyridoxal 5-phosphate	247.02	2.76	x	x
Pyridoxine	169.07	1.79	x	x
Pyrrole-2-carboxylate	111.03	4.71		x
Pyruvic aldehyde	72.021	2.20		x
Quinate	192.06	1.47	x	x
Quinoline	129.06	6.05	x	
Rac-glycerol 1-myristate	302.25	13.53	x	x
Reichstein's substance S	346.21	9.98	x	x
Resorcinol monoacetate	152.05	7.94		x
Retinoate	300.21	13.2	x	x
Retinol	286.23	9.02	x	
Ribitol	152.07	1.36	x	x
Riboflavin	376.14	6.50	x	x
Rosmarinic acid	360.08	7.35	x	x
S-(5-Adenosyl)-L-homocysteine	384.12	2.53	x	x
Salicylamide	137.05	6.08	x	x
Salicylate	138.03	6.91	x	x
Sarcosine	89.048	1.30	x	x
S-Carboxymethyl-L-cysteine	179.03	1.43	x	x
Selenocystamine	235.95	1.91		x
Selenomethionine	191	2.18		x
Serotonin	176.1	1.56	x	
S-Hexyl-glutathione	391.18	8.77	x	x
Shikimate	174.05	1.65		x
Sn-Glycerol 3-phosphate	172.01	1.43	x	x
Sorbate	112.05	9.28		x
Spermine	202.22	1.50	x	
Sphinganine	301.3	12.21	x	x
Sphingomyelin	730.6	14.01		x
Stachyose	666.22	1.39	x	x
Stearate	284.27	13.88		x
Suberic acid	174.09	7.10	x	x
Succinic acid	118.03	2.39		x
Sucrose	342.12	1.44	x	x
Taurine	125.01	1.27	x	x
Taurolithocholate	483.3	11.95	x	x
Theobromine	180.06	4.55	x	
Theophylline	180.06	5.23	x	x

Thiamine monophosphate	344.07	1.22	x	
Thiamine pyrophosphate	424.04	1.42	x	x
Thiopurine-s-methylether	166.03	6.12		x
Thiourea	76.01	1.46		x
Thymidine	242.09	3.96	x	x
Thymidine 5-monophosphate	322.06	2.80	x	
Thymidine-5-diphospho-α-D-glucose	564.08	2.25	x	x
Thymine	126.04	3.05	x	x
Thyrotropin releasing hormone	362.17	2.12	x	
Thyroxine	776.69	10.13	x	
Trans-4-Hydroxyproline	131.06	1.44	x	x
Trans-Aconitate	174.02	2.55		x
Trans-Cinnamaldehyde	148.05	8.74	x	x
Trans-Cinnamate	148.05	8.69	x	
Trans-Cyclohexane-1,2-diol	116.08	5.22	x	
Trigonelline	137.05	1.40	x	x
Tryptamine	160.1	5.90		x
Tyramine	137.08	2.29	x	
Uracil	112.03	1.81	x	x
Urate	168.03	1.90		x
Uridine	244.07	2.26	x	x
Uridine 5-diphosphogalactose	566.06	1.64	x	x
Uridine 5-diphosphoglucose	566.06	1.66	x	x
Uridine 5-diphospho-N-acetylgalactosamine	607.08	1.71		x
Uridine 5-diphospho-N-acetylglucosamine	607.08	1.70		x
Uridine-5-monophosphate	324.04	1.73	x	x
Urocanate	138.04	1.61	x	x
Xanthine	152.03	2.27	x	x
Xanthosine	284.08	3.49	x	x
Xanthurenic acid	205.04	5.03	x	
Xylitol	152.07	1.36	x	x
α-Aminoadipate	161.07	1.38	x	x
α-D-Galactose 1-phosphate	260.03	1.33	x	x
α-D-Glucose	180.06	1.34	x	x
α-D-Glucose 1-phosphate	260.03	1.40	x	x
α-Hydroxyisobutyric acid	104.05	3.20		x
α-Ketoglutaric acid	146.02	1.67		x
β-Alanine	89.048	1.24		x
β-Carotene	536.44	13.81	x	x
Bine	117.08	1.33	x	x
Ω-Hydroxydodecanoic acid	216.17	10.52	x	x

Supplementary Table 2.3. Method 2 accurate mass retention time data from the analysis of mass spectrometry metabolite library of standards. Supplied by IROA Technologies, LLC. Product information provided by Sigma-Aldrich (Version PD, MAM 09/15-1). RT – retention time; +VE – positive; -VE – negative; x – detected in respective polarity.

Compound name	Mass	RT	Polarity	
			+VE	-VE
(2-Aminoethyl)phosphonate	125.024	8.48	x	
(R)-Malate	134.022	4.51		x
(S)-Dihydroorotate	158.033	4.71		x
(S)-Malate	134.022	4.51		x
1,2-Didecanoyl-Sn-glycero-3-phosphocholine	565.374	4.35	x	
1,2-Dipalmitoyl-rac-glycero-3-phosphoethanolamine	691.515	4.23	x	x
10-Hydroxydecanoate	188.141	1.70		x
1-Hydroxy-2-naphthoate	188.047	1.70		x
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	179.095	4.42	x	x
1-Methyladenosine	281.112	6.18	x	x
1-Oleoyl-rac-glycerol	356.293	1.64	x	
2,3-Dihydroxybenzoate	154.027	1.73		x
2,4-Dihydroxypteridine	164.033	4.15		x
2,4-Dihydroxypyrimidine-5-carboxylic acid	156.017	3.86		x
2,5-Dihydroxybenzoate	154.027	1.73		x
2-Amino-2-methylpropanoate	103.063	6.19	x	
2-Aminophenol	109.053	1.74	x	x
2-Deoxyadenosine	251.102	4.99	x	x
2'-Deoxyadenosine 5'-triphosphate	251.102	4.64		x
2-Deoxycytidine 5-monophosphate	307.057	8.51	x	x
2-Deoxyguanosine	267.097	5.95	x	x
2-Deoxyguanosine 5-monophosphate	347.063	8.63	x	x
2-Deoxyuridine 5-mono-Phosphate	308.041	8.35		x
2-Hydroxy-4-(methylthio)butyric acid	150.035	1.73		x
2-Hydroxybutyric acid	104.047	2.15		x
2-Hydroxyphenylacetic acid	152.047	1.72		x
2-Hydroxypyridine	95.0371	5.63		x
2-Methylmaleate	130.027	4.0		x
2-Oxoadipate	160.037	3.78		x
2-Quinolinecarboxylic acid	173.048	1.70	x	
3-(2-Hydroxyphenyl)propanoate	166.063	1.81	x	
3-(4-Hydroxyphenyl)lactic acid	182.058	1.75		x
3-(4-Hydroxyphenyl)pyruvic acid	180.04	3.80		x
3,3,5-Triiodothyronine	650.79	4.92	x	x
3,4-Dihydroxybenzoate	154.027	1.76		x
3,4-Dihydroxy-L-phenylalanine	197.069	6.67	x	x
3,4-Dihydroxyphenyl glycol	170.058	4.07		x
3,4-Dihydroxyphenylacetate	168.042	1.74		x
3,5-Cyclic adenosine monophosphate	329.053	7.80		x

3,5-Diiodo-L-thyronine	524.893	5.00	x	
3,5-Diiodo-L-tyrosine	432.867	5.58	x	x
3-Amino-4-hydroxybenzoic acid	153.043	1.74	x	x
3-Amino-5-hydroxybenzoic acid	153.043	1.75	x	
3-Aminoisobutanoate	103.063	5.34	x	
3-Dehydroshikimate	172.037	4.15		x
3-Hydroxy-3-methylglutarate	162.053	3.88		x
3-Hydroxyanthranilate	153.043	1.72	x	x
3-Hydroxybenzaldehyde	122.037	1.55		x
3-Hydroxyphenylacetate	152.047	1.74	x	
3-Methoxy-4-hydroxymandelate	198.053	3.54		x
3-Methoxy-L-tyrosine	211.085	6.14	x	x
3-Methoxytyramine	167.095	3.51	x	
3-Methyglutaric acid	146.058	1.73		x
3-Methyl-2-oxindole	147.068	1.71		x
3-Methyl-2-oxovaleric acid	130.063	1.64		x
3-Methyladenine	149.07	4.79	x	
3-Methylhistamine	125.095	6.78	x	
3-Nitro-L-tyrosine	226.059	5.87	x	x
3-Ureidopropionate	132.054	4.50	x	
3α,12α-Dihydroxy-5β-cholanate	392.293	1.50	x	
4-Acetamidobutanoate	145.074	3.63		x
4-Aminobenzoate	137.048	1.70	x	
4-Aminobutanoate	103.063	5.40	x	x
4-Coumarate	164.047	1.72		x
4-Guanidinobutanoate	145.085	4.96	x	x
4-Hydroxy-2-quinolinecarboxylic acid	189.043	5.02	x	x
4-Hydroxy-L-proline	131.058	6.98	x	x
4-Hydroxyphenylacetic acid	152.047	1.73	x	
4-Imidazoleacetic acid	126.043	5.33	x	
4-Methyl-2-oxo-pentanoic acid	130.063	1.74		x
4-Methyl-2-oxovaleric acid	130.063	1.64		x
4-Methylcatechol	124.052	1.78		x
4-Quinolinecarboxylic acid	173.048	2.0	x	x
5,6-Dihydrouracil	114.043	5.84	x	
5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5-monophosphate	338.063	8.76	x	x
5-Aminolevulinic acid	131.058	5.48	x	
5-Aminopentanoate	117.079	5.02	x	
5-Cytidine monophosphate	323.052	8.77		x
5-Deoxyadenosine	251.102	4.64	x	x
5-Hydroxyindoleacetate	191.058	1.73	x	x
5-Hydroxy-L-tryptophan	220.085	6.27	x	x

5-Hydroxymethyluracil	142.038	4.99		x
5-Methylcytosine hydrocloride	125.059	5.52	x	
5-Methylthioadenosine	297.09	4.47	x	x
5-Oxo-D-proline	129.043	4.14	x	x
5-Oxo-L-proline	129.043	4.14	x	x
5-Valerolactone	100.052	1.70	x	
6-Carboxyhexanoate	160.074	1.72	x	x
6-Deoxy-L-galactose	164.069	5.77	x	
6-Hydroxynicotinate	139.027	4.30	x	x
Adenine	135.055	4.48	x	x
Adenine hydrochloride hydrate	135.055	5.23		x
Adenosine	267.097	5.37	x	x
Adenosine 2,3-cyclic monophosphate	329.053	7.67	x	x
Adenosine 3,5-cyclic monophosphate	329.053	7.80	x	x
Adenosine 5-diphosphoribose	559.071	11.44	x	x
Adenosine 5-monophosphate	347.063	8.63	x	x
Adenosine-5-diphosphoglucose	589.082	11.10		x
Adipic acid	146.058	1.73		x
Agmatine sulfate	130.122	6.93	x	
Allantoin	158.044	5.49		x
Allose	180.063	6.62		x
Aniline	93.0578	5.04	x	
Anthranilate	137.048	1.69	x	
Arabinose	150.053	6.11	x	x
Arachidic Acid	312.303	8.26	x	
Azelaic acid	188.105	1.71		x
Bilirubin	584.264	2.08	x	
Biliverdin	582.248	4.48	x	
Biotin	244.088	3.73	x	x
Bis(3-aminopropyl)amine	131.142	8.76	x	
Cadaverine	102.116	6.96	x	
Caffeate	180.042	1.73	x	x
Caffeine	194.08	1.66	x	
Carnosine	226.107	7.98	x	x
Catechol	110.037	1.76		x
Chenodeoxycholate	392.293	1.72		x
Cholate	408.288	1.75	x	
Cis-4-Hydroxy-D-proline	131.058	6.91	x	x
Citramalate	148.037	4.34		x
Citrulline	175.096	7.46	x	x
Coenzyme Q10	862.684	8.74	x	
Corticosterone	346.214	1.65	x	x
Cortisol	362.209	1.71	x	

Cortisol 21-acetate	404.22	1.63	x	x
Cortisone	360.194	1.70	x	x
Creatine	131.07	5.84	x	
Creatinine	113.059	4.99	x	x
Cys-Gly	178.041	8.55	x	
Cytidine	243.086	6.46	x	x
Cytidine 2,3-cyclic mono-phosphate	305.041	8.07	x	x
Cytidine 5-diphosphocholine	488.107	9.39	x	x
Cytosine	111.043	5.84	x	
D-(+)-Cellobiose	342.116	7.88	x	x
D-(+)-Galacturonic acid	194.043	7.12		x
D-(+)-Glucosamine	179.079	7.55	x	
D-(+)-Raffinose	504.169	8.65	x	x
D-(+)-Trehalose	342.116	8.10	x	x
D-Alanine	89.0477	6.32	x	x
D-AMP	331.068	8.59	x	x
D-Aspartate	133.038	7.28	x	x
Decanoate	172.146	1.64	x	
Deoxycarnitine	145.11	3.55	x	
Deoxycholate	392.293	1.73		x
Deoxycytidine	227.091	6.10		x
D-Fructose 6-phosphate	260.03	9.30	x	x
D-Galactose	180.063	7.04		x
D-Gluconate	196.058	6.83		x
D-Glucono-1,5-lactone	196.058	6.81		x
D-Glucosamine 6-phosphate	259.046	9.45		x
D-Glucuronic acid	194.043	6.80		x
D-Glucuronolactone	194.043	6.77		x
D-Glyceric acid	106.027	4.33		x
Diethanolamine	105.079	5.77	x	
Diethyl 2-methyl-3-oxosuccinate	202.084	1.70	x	
Dihydroxymandelic acid	184.037	4.34		x
Dimethylbenzimidazole	146.084	1.78		x
DL-5-Hydroxylysine	162.10	8.31	x	x
D-Lactose	342.116	8.06		x
DL-Kyneurenine	208.085	5.79	x	
D-Lyxose	150.053	5.59	x	
D-Mannosamine	179.079	7.18	x	
D-Mannose 6-phosphate	260.03	9.47	x	x
Dopamine	153.079	5.23	x	x
D-Ornithine	132.09	8.19		x
D-Pantothenic acid	219.111	3.98	x	x
D-Ribose	150.053	4.99	x	

D-Sorbitol	182.079	6.63		x
D-Tagatose	180.063	6.32	x	
D-Tryptophan	204.09	5.67	x	x
D-Xylose	150.053	5.57	x	
Elaidic acid	282.256	1.64	x	
Epinephrine	183.09	5.23	x	x
Flavin adenine dinucleotide	785.157	11.46		x
Folic acid	441.14	5.98	x	x
Formyl-L-methionyl peptide	177.046	3.44		x
Galactitol	182.079	6.74		x
γ–Linoleic acid	278.225	1.70	x	
Gluconic acid	196.058	6.95		x
Glucosaminate	195.074	8.31	x	x
Glutathione	307.084	7.05	x	x
Glycerate	106.027	4.31		x
Glycocholate	465.309	4.48	x	x
Guanidinoacetate	117.054	6.21	x	
Guanine	151.049	6.06	x	
Guanosine	283.092	6.39	x	x
Guanosine 3,5-cyclic monophosphate	345.047	8.28	x	x
Guanosine 5-diphosphoglucose	605.077	12.95		x
Hippurate	179.058	1.72	x	
Histamine	111.08	7.09	x	
Homocystine	268.055	8.25	x	x
Homogentisate	168.042	1.78		x
Homoserine	119.058	6.98	x	x
Hypotaurine	109.02	7.23	x	
Hypoxanthine	136.039	5.12	x	x
Indole-3-acetaldehyde	159.068	5.62		x
Indole-3-acetamide	174.079	1.70	x	
Indole-3-acetic acid	175.063	1.70	x	
Indole-3-pyruvic acid	203.058	1.70	x	
Indoxyl sulfate	213.01	4.22		x
Inosine	268.081	5.74	x	x
Inosine 5'-phosphate	348.047	9.02	x	
L-2,6-Diaminoheptanedioate	190.095	8.8	x	x
L-Alanine	89.0477	6.38	x	
L-Allothreonine	119.058	6.99		x
L-Anserine	240.122	7.57	x	x
L-Arabitol	152.069	6.04		x
L-Arginine	174.112	7.98	x	x
L-Asparagine	132.054	7.50	x	x
L-Aspartate	133.038	7.27	x	x

Lauroylcarnitine	343.272	1.71	x	x
L-Carnitine	161.105	4.49	x	
L-Cystathionine	222.067	8.80	x	x
L-Cysteic acid	169.005	8.23	x	x
L-Cystine	240.024	8.99	x	x
Leucine	131.095	5.42	x	
L-Glutamic acid	147.053	6.95	x	x
L-Glutamine	146.069	7.31	x	x
L-Histidine	155.07	8.08	x	
L-Histidinol	141.09	7.33	x	x
L-Homocysteine thiolactone	117.025	4.61	x	
Lipoamide	205.06	1.64	x	
L-Isoleucine	131.095	5.59	x	x
L-Kynurenine	208.085	5.79	x	
L-Lysine	146.106	8.10	x	x
L-Lysine monohydrochloride	146.106	8.08	x	x
L-Methionine	149.051	6.06	x	
L-Methionine sulfoximine	180.057	7.59	x	x
L-Norvaline	117.079	5.84	x	
L-Ornithine	132.09	8.06	x	x
L-Phenylalanine	165.079	5.68	x	x
L-Phosphatidylcholine	757.562	4.37	x	
L-Pipecolic acid	129.079	6.29	x	
L-Proline	115.063	6.31	x	
L-Serine	105.043	7.37	x	x
L-Sorbose	180.063	6.43		x
L-Threonine	119.058	6.98		x
L-Tryptophan	204.09	5.68	x	x
L-Tryptophanamide	203.106	4.60	x	
L-Tyrosine	181.074	6.26	x	x
Lumichrome	242.08	1.80	x	x
L-Valine	117.079	6.01	x	
Maleic acid	116.011	4.17		x
Maltose	342.116	7.63	x	x
Mandelic acid	152.047	1.72		x
Mannitol	182.079	6.66		x
Mannose	180.063	6.52		x
Melatonin	232.121	1.65	x	
Melibiose	342.116	8.33	x	x
Methyl indole-3-acetate	189.079	1.71	x	
Methyl vanillate	168.042	1.69		x
Methyl β-D-galactoside	194.079	5.37	x	x
Methylmalonate	118.027	3.72	x	x

Mono-methyl glutarate	146.058	1.72		x
Myoinositol	180.063	8.22		x
N(Pai)-Methyl-L-histidine	169.085	7.64	x	
N,N-Dimethyl-1,4-phenylenediamine	136.10	1.70	x	
N1-Acetylspermine	244.226	8.53	x	
N-6-(δ2-isopentenyl)-adenine	203.117	1.74	x	x
N-Acetyl-D-galactosamine	221.09	6.15		x
N-Acetyl-D-glucosamine	221.09	6.24		x
N-Acetyl-DL-glutamic acid	189.064	4.40	x	x
N-Acetyl-DL-methionine	191.062	2.85		x
N-Acetyl-DL-serine	147.053	4.39		x
N-Acetyl-D-mannosamine	221.09	6.19		x
N-Acetyl-D-tryptophan	246.10	3.56	x	x
N-Acetylglycine	117.043	3.75		x
N-Acetyl-L-alanine	131.058	3.34		x
N-Acetyl-L-aspartic acid	175.048	4.54		x
N-Acetyl-L-cysteine	163.03	3.49		x
N-Acetyl-L-leucine	173.105	1.72	x	x
N-Acetyl-L-phenylalanine	207.09	1.71		x
N-Acetylneuraminate	309.106	7.02	x	x
N-Acetylputrescine	130.111	5.28	x	
Nicotinamide adenine dinucleotide	663.109	9.73	x	
N-Amidino-L-aspartate	175.059	6.40	x	x
N-Formylglycine	103.027	3.75		x
Nicotinamide	122.048	3.51	x	
Nicotinamide hypoxanthine dinucleotide	664.093	10.13	x	x
Nicotinamide mononucleotide	334.057	8.37	x	
Nicotinate	123.032	2.79	x	x
N-Methyl-D-aspartic acid	147.053	6.82	x	x
N-Methyl-L-glutamate	161.069	6.58	x	x
Noradrenaline	169.074	6.07		x
Norleucine	131.095	5.31	x	
N-α-Acetyl-L-asparagine	174.064	4.88	x	x
N-α-Acetyl-L-lysine	188.116	6.06	x	x
Nε, Nε, Nε -Trimethyllysine	188.153	7.38	x	x
O-Acetyl-L-serine	147.053	6.37	x	
Orotic acid	156.017	6.46		x
O-Succinyl-L-homoserine	219.074	6.37	x	
Palatinose	342.116	7.76	x	x
Paraxanthine	180.065	3.53	x	
Petroselinic acid	282.256	1.64	x	
Phenylpyruvate	164.047	1.65		x
Phospho(enol)pyruvic acid	167.982	8.46	x	

Phosphocholine	183.066	8.26	x	
Phylloquinone	450.35	1.71	x	
Pipecolate	129.079	6.25	x	
Protoporphyrin	562.258	3.57	x	x
Pterin	163.049	5.23	x	
Purine	120.044	4.13	x	x
Pyridoxal	167.058	4.32		x
Pyridoxal 5-phosphate	247.025	7.52	x	x
Pyridoxamine	168.09	6.66	x	
Pyridoxine	169.074	5.0	x	x
Quinate	192.063	6.06		x
Quinoline	129.058	1.65	x	
Reichstein's substance S	346.214	1.65	x	x
Resorcinol monoacetate	152.047	1.51		x
Retinoate	300.209	1.50	x	
Retinyl palmitate	524.459	1.56	x	
Ribitol	152.069	5.88		x
Riboflavin	376.138	5.60	x	x
Rosmarinic acid	360.085	4.17	x	x
S-(5-Adenosyl)-L-homocysteine	384.122	7.66	x	x
S-(5-Adenosyl)-L-methionine	398.137	8.36	x	
Salicylate	138.032	1.70		x
Sarcosine	89.0477	6.47	x	x
S-Carboxymethyl-L-cysteine	179.025	6.99	x	
Selenocystamine	235.945	6.95	x	
Selenomethionine	191.002	5.99	x	
Serotonin	176.095	4.51	x	
S-Hexyl-glutathione	391.178	5.82	x	x
Shikimate	174.053	4.99		x
Spermidine	145.158	8.63	x	
Spermine	202.216	9.70	x	
Sphinganine	301.298	1.73	x	
Sphingomyelin	730.599	4.75	x	
Stachyose	666.222	9.38	x	x
Suberic acid	174.089	1.72		x
Succinic acid	118.027	3.61		x
Sucrose	342.116	7.63	x	x
Taurine	125.015	6.79	x	x
Taurolithocholate	483.302	5.04	x	x
Theophylline	180.065	3.40		x
Thiamine monophosphate	344.071	8.35	x	
Thiopurine S-methylether	166.031	1.73	x	x
Thymidine	242.09	4.21		x

Thymidine 5-monophosphate	322.057	8.08		x
Thymidine-5'-diphospho-α-D-glucose	564.076	12.53		x
Thymine	126.043	3.85		x
Thyrotropin releasing hormone	362.17	6.49	x	x
Thyroxine	776.687	5.00		x
Trans-4-Hydroxyproline	131.058	7.00	x	x
Trans-Aconitate	174.016	4.44		x
Trans-Cinnamate	148.052	1.70	x	
Tryptamine	160.1	1.74	x	
Tyramine	137.084	3.42	x	
Uracil	112.027	4.05		x
Urate	168.028	6.62	x	x
Uridine	244.07	5.11		x
Uridine 5-diphosphogalactose	566.055	12.95		x
Uridine 5-diphosphoglucose	566.055	12.68		x
Uridine 5-diphospho-N-acetylgalactosamine	607.082	12.33		x
Uridine 5-diphospho-N-acetylglucosamine	607.082	12.06		x
Uridine-5-monophosphate	324.036	9	x	x
Urocanate	138.043	4.88		x
Vitamin B12	1354.57	7.65	x	x
Xanthine	152.033	5.45	x	x
Xanthosine	284.076	5.96	x	x
Xanthurenic acid	205.038	5.18	x	x
Xylitol	152.069	5.96		x
α-Aminoadipate	161.069	6.65	x	x
α-D-Galactose 1-phosphate	260.03	9.61	x	x
α-D-Glucose	180.063	6.87		x
α-D-Glucose 1-phosphate	260.03	9.5		x
β-Alanine	89.0477	6.1	x	
Bine	117.079	6.03	x	
Ω-Hydroxydodecanoic acid	216.173	1.66		x

Supplementary Table 2.4. Method 3 accurate mass retention time data from the analysis of mass spectrometry metabolite library of standards. Supplied by IROA Technologies, LLC. Product information provided by Sigma-Aldrich (Version PD, MAM 09/15-1). RT – retention time ; +VE – positive; -VE – negative; x – detected in respective polarity.

Compound	Fold change	Abundance		p value
		Up	Down	
Acetyl-tyrosine[1]	9.3	√		<0.0001
γ-Glutamyl-tyrosine[1]	16	√		<0.0001
2,5-Dihydroxybenzaldehyde[1]	14.1		√	<0.0001
Hydroxynitisinone[5]	100+	√		<0.0001

Supplementary Table 2.5. Non-AMRT urinary metabolite changes identified post-nitisinone. Fold changes (FC's) for patients with AKU are indicated in brackets and were calculated from raw peak area. Compounds were identified by theoretical accurate mass match <10 ppm. **Bold** text indicates change observed in human and mouse data. Metabolic pathway affected denoted by a number: 1 – tyrosine metabolism; 5 – nitisinone.

2.8 Declaration and acknowledgements

Preparation, analysis and data processing associated with IROA analytical standards and urine samples from patients attending the NAC was performed by Andrew Davison and Brendan Norman (Department of Musculoskeletal Biology I, University of Liverpool, UK).

Preparation, analysis and data processing associated with mouse urine samples was performed by Brendan Norman (Department of Musculoskeletal Biology I, University of Liverpool, UK).

Hazel Sutherland (Department of Musculoskeletal Biology I, University of Liverpool, UK) performed the mouse dosing and collection of urine samples. The mice in these studies were housed under the licence of Jonathan Jarvis (Department of Exercise and Sports Science, Liverpool John Moores University, Liverpool, UK).

Jean Devine (Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Royal Liverpool Hospital, Liverpool, UK) processed all urine samples from patients attending the NAC.

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Chapter 3

Evaluation of the serum metabolome of patients with alkaptonuria before and after 2 years of treatment with nitisinone using LC-QTOF-MS

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3.1 Abstract

Background: The homogentisic acid-lowering therapy nitisinone is being evaluated for the treatment of Alkaptonuria (AKU) at the National Centre for AKU. Beyond hypertyrosinaemia the wider metabolic consequences of its use are largely unknown. The aim of this work was to evaluate the impact of nitisinone on the serum metabolome of patients with AKU after 12 and 24 months of treatment.

Materials and methods: Deproteinised serum from 25 patients with AKU (mean age±SD 51.1±14.9 y, 12 male) was analysed using the 1290 Infinity II LC system coupled to a 6550 QTOF-MS (Agilent, Cheadle, UK). Raw data were processed using a batch targeted feature extraction algorithm and an accurate mass retention time database containing 469 intermediary metabolites (mw 72-785). Matched entities (± 10ppm theoretical accurate mass and ± 0.3 min retention time window) were filtered based on their frequency and variability (<25 % CV) in group QC samples, and repeated measures statistical significance analysis with Benjamini-Hochberg false discovery rate adjustment was used to assess changes in metabolite abundance.

Results: 8 metabolites increased in abundance (log_2 fold change 2.1-15.2, $p<0.05$); 7 of 8 entities were related to tyrosine metabolism, and 13 decreased in abundance (log_2 fold change 1.5-15.5, $p<0.05$); including entities related to tyrosine (n=2); tryptophan (n=3); xanthine (n=2) and citric acid cycle metabolism (n=2).

Conclusions: Evaluation of the serum metabolome of patients with AKU showed a significant difference in the abundance of several metabolites following treatment with nitisinone, including a number that have not been previously reported; several of these were not related to the tyrosine metabolic pathway.

3.2 Introduction

Alkaptonuria (AKU, OMIM 203500) is a rare autosomal recessive disorder of tyrosine metabolism resulting from a defect in homogentisate-1,2-dioxygenase (HGD, E.C.1.12.11.5), which leads to a marked increase in the circulating concentration of homogentisic acid (HGA) (Phornphutkul *et al.,* 2002). The pathological hallmark of AKU is 'ochronosis', which is a consequence of the deposition of a dark pigment in connective tissue, mainly cartilage, which alters its physico-mechanical properties. The exact composition and structure of this pigment is unknown, but is known to result from the accumulation of HGA (Figure S3.1) (Ranganath *et al.,* 2013).

Treatment options for AKU are conservative in large focusing on supportive and palliative measures (Ranganath *et al.,* 2013). The HGA lowering agent nitisinone (Figure S3.1) has been shown to completely prevent ochronosis in AKU mice (Preston *et al.,* 2014) and is being evaluated as a potential treatment in AKU patients (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Ranganath *et al.,* 2018); it is however not without its own challenges as it is well documented to result in significant hypertyrosinaemia in AKU (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al*. 2017; Ranganath *et al.,* 2018; Davison *et al.,* 2018a; Davison *et al.,* 2018b; Davison *et al.,* 2018c) and hereditary tyrosinaemia type 1 (HT1) (Lindstedt *et al.,* 1992; Lock, 2017; van Ginkel *et al.,* 2017). The consequences of this are largely unknown in AKU; in HT1 it has been suggested this may contribute to the neurodevelopmental delay that is frequently observed in children treated with nitisinone (McKiernan *et al.,* 2015). Several mechanisms have been proposed for this including altered metabolism of the monoamine neurotransmitters (Thimm *et al.,* 2011). Davison *et al.* (2018b; 2018c) demonstrated in a cohort of AKU patients that nitisinone therapy resulted in altered urinary excretion of dopaminergic and serotoninergic neurotransmitter metabolites. However, these findings are limited as they are not a direct reflection of neurotransmitter metabolism in the central nervous system. A

recent study in an animal model of HT1 suggested that the disease itself and not treatment with nitisinone is likely to be responsible for slower learning and altered behavior in mice (Hillgartner *et al.,* 2016).

Recently, changes in the urine metabolome of an AKU mouse model and patients with AKU treated with nitisinone were reported (Norman *et al.,* 2019a). This study demonstrated novel changes in the tyrosine metabolic pathway, and unexpectedly in tryptophan and purine metabolism. While these changes in the urine metabolome provide insight into how nitisinone alters metabolism, one may postulate that changes observed in the serum are more relevant as they are a more direct reflection of internal homeostasis. Gertsman *et al.* (2015a) reported on the impact of nitisinone therapy on the serum metabolome of patients with AKU. In addition to the expected decrease in HGA and increase in tyrosine, significant increases in N-acetyl-L-tyrosine and γ-glutamyltyrosine were also observed. In a separate publication (Gertsman *et al.,* 2015b) in the same cohort of patients, novel disturbances in tryptophan metabolism were reported.

Herein for the first time we report the impact of nitisinone (2 mg daily) therapy on the serum metabolome in the largest cohort of AKU patients to date over a 2 year period at the National AKU Centre (NAC) in the UK.

3.3 Materials and methods

3.3.1 Reagents

Water for mobile phases was purified in-house (DIRECT-Q 3UV Millipore water purification system). Methanol, acetonitrile and isopropanol were purchased from Sigma Aldrich (Dorset, UK). Formic acid and ammonium formate were obtained from Biosolve, (Netherlands) and Fisher Scientific (Germany), respectively. All reagents were LC-MS grade. N-Acetyl-L-tyrosine was purchased from Sigma Aldrich, UK.

3.3.2 Patients and serum sample collection

3.3.2.1 Ethical Approval

Data collection and sample analyses at the NAC has approval from the Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (Audit no. ACO3836), this approval includes the use of patient data and biological material for metabolomics evaluation. As data and samples were collected as part of the clinical service, ethical approval was not required. Patients are informed verbally and through patient information leaflets about the clinical and research activities of the NAC and are informed that data may be used for publication. For a detailed protocol see Milan *et al.* (2017).

3.3.2.2 Sample collection

Serum samples (S-monovette, Sarstedt, Germany) were collected from patients after an overnight fast (≥8 h). Patients' dietary intake of protein was managed through a 7-day food diary by a combination of lower protein in diet and phenylalanine/tyrosine-free meal exchanges. Samples were centrifuged at 1500 ×*g* for 10 min at 4 °C; and then deproteinised with perchloric acid (60 % 5.8 M; 1:10, perchloric acid:serum), vortexed and centrifuged at 1500 ×*g* for 10 min. Supernatant was stored at -20 °C until analysis.

3.3.2.3 Patient and quality control sample preparation

Patient samples were prepared by diluting 150 *µ*L serum with 450 *µ*L deionized water (DIRECT-Q 3UV Millipore water purification system). Diluted samples were then transferred into a 96 well plate which was then agitated on a plate shaker (MTS 2/4m IKA, Germany) at 600 rpm for 10 min.

Patient group quality control (QC) samples were produced by adding 50 *µ*L of each patient sample into a single pool. In total 4 group QC pools were made [(i) baseline, (ii) 12 months, (iii) 24 months and (iv) overall – containing serum from all patients and visits; this acted as a system QC]. QC samples were prepared as per patient samples.

The analytical sequence of samples was performed as per published guidance (Vorkas *et al.,* 2015). Each run commenced with 20 replicate

injections of the overall pooled sample to condition the system. The order of individual samples was randomised computationally. Pooled samples were interspersed throughout the analytical sequence every tenth injection.

3.3.3 Analytical method

3.3.3.1 Chromatographic conditions

Liquid chromatography (LC) was performed on an Agilent 1290 Infinity II LC system. An Atlantis dC_{18} column (3.0 x 100 mm, 3 μm, Waters, UK) was maintained at 60 °C with a flow rate of 0.4 mL/min. Mobile phases were (A) water and (B) methanol both containing 5 mmol/L ammonium formate and 0.1 % formic acid. The elution gradient started at 5 % mobile phase B at 0-1 min increasing linearly to 100 % B by 12 min, held at 100 % B until 14 min, returning to 95 % A for 5 min to recondition the column. Injection volume was 1 μL.

3.3.3.2 Quadrupole time-of-flight mass spectrometry (QTOF-MS) conditions

An Agilent 6550 QTOF-MS equipped with a dual jet stream electrospray ionisation source was operated in 2 GHz mode, over the mass range of 50-1700, in negative and positive polarities. A reference mass correction solution was continually infused at a flow rate of 0.5 mL/min via an external isocratic pump (Agilent, Cheadle, UK) for constant mass correction (see section 3.7 Supplementary material for additional details of QTOF-MS operating parameters and composition of reference ion solution).

3.3.3.3 Metabolite identification, data quality control and statistical analyses

Metabolite identification was carried out using an established accurate mass retention time (AMRT) database to match chemical entities (Norman *et al.,* 2019a). The database included theoretical accurate mass, measured retention time, and empirical formula. This was modified to include N-acetyl-L-tyrosine, γ-glutamyl-tyrosine and indole-3-lactate (I-3-L). AMRT data for N-acetyl-L-tyrosine were verified following the analysis of an analytical standard. AMRT data for γ-glutamyl-tyrosine and I-3-L were based on the

elution time associated with theoretical monoisotopic mass of each compound and were not verified using an analytical standard. Data quality control and statistical analysis were performed using the MassHunter software suite (Agilent, Cheadle, UK). For additional details on data quality control and statistical analyses see supplementary information 3.1 and Supplementary Figure S3.2 and S3.3.

Serum samples were also analysed using 1D proton (^{1}H) nuclear magnetic resonance (NMR). Data from these analyses were not included in the peer reviewed publication presented herein. For details of sample preparation, analytical methodology, data analysis, results and discussion see Appendix 1 at the end of this chapter.

3.4 Results

Twenty-five patients [13 female, mean age (±standard deviation) 55.3(15.3) years (range 22-75); 12 male, mean age 44.2(15.8) years (range 22-70)] were included at baseline and after treatment with nitisinone at 12 and 24 months.

Raw data from LC-QTOF-MS analysis showed that retention time and accurate mass ranges were 1.15-13.93 min and 75.0318-730.5955 Da, in positive and 1.03-14.67 min and 75.0318-722.6247 Da in negative polarity, respectively. Principal component analysis and associated loading plots in positive and negative polarities are depicted in Figure 3.1. Clear separation between the AMRT matched profiles of AKU patients pre- and post-nitisinone therapy was observed in component 2 in positive polarity. Negative polarity shows poor separation in comparison. Quantitative statistical analysis was performed to further evaluate changes in metabolite abundance following treatment with nitisinone.

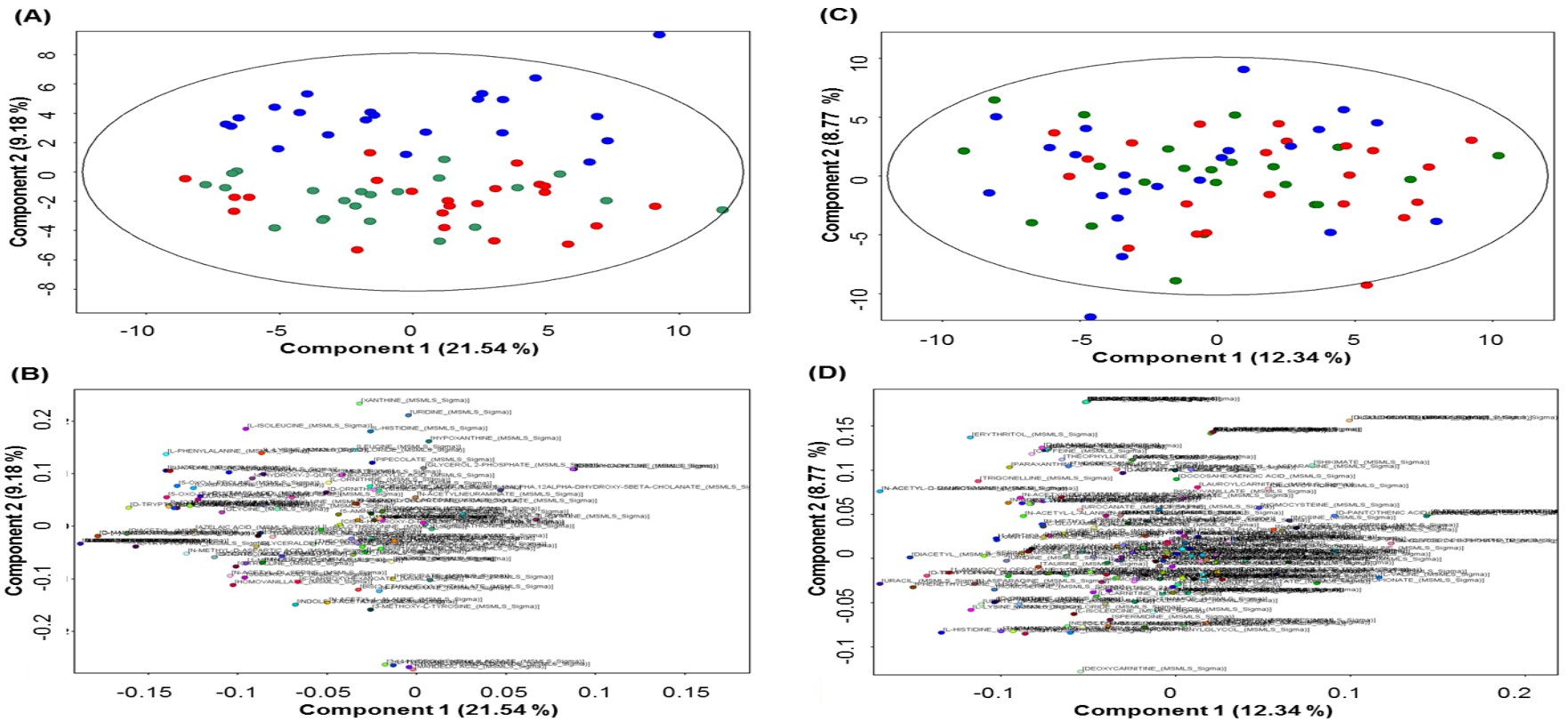

Figure 3.1. **Principal component analysis to assess the effect of nitisinone therapy on metabolite profiles in serum samples collected from AKU patients.** Baseline (no treatment) – blue circle; 12 months (2 mg daily nitisinone) – green circle and 24 months (2 mg daily nitisinone) – red circle. PCA plots (A) in positive and (C) negative polarity. Loadings plots (B) and (D) show the respective contributions of individual metabolites to components 1 and 2 in positive and negative polarity, respectively.

151/469 and 249/469 metabolites were aligned across all samples in positive and negative polarities (for matched compounds see Table S3.2 and Supplementary Figure S3.4), respectively at baseline, and after nitisinone treatment at 12 and 24 months. After filtering entities based on their frequency and variability across replicate injections of pooled QC samples from each experimental group, 123 and 209 entities were retained from positive and negative polarity profiling experiments, respectively. Of these, 60 and 121 respectively, were shown to be significantly different ($p<0.05$) following nitisinone therapy. Those with a $\log_2$ fold change (FC) >2 at 12 and or 24 months are summarised in Table 3.1. Applying this cut-off, 8 (6.5 %) entities were considered to increase in abundance and 13 (10.7 %) decrease in abundance.

Compound	Log_2 FC		p value	Abundance	
	12 months	24 months		Down	Up
Glycocholate[4]	12.7	12.7	<0.001	√	
Succinic acid[6]	11.4	11.4	<0.001	√	
α-Ketoglutaric acid[6]	10.0	10.0	<0.001	√	
Trans-4-hydroxyproline[4]	15.5	15.5	<0.001	√	
Homoserine[4]	12.5	12.5	<0.001	√	
Mevalolactone[4]	10.7	10.7	<0.001	√	
L-Allothreonine[4]	11.8	11.8	0.02	√	
Trigonelline[2]	12.8	12.8	<0.001	√	
4-Quinolinecarboxylic acid[2]	8.3	8.3	<0.001	√	
Indole-3-lactate[2]	2.1	2.3	<0.001		√
4-Hydroxyphenylacetic acid[1]	4.2	5.4	<0.001		√
Benzaldehyde[1]	6.9	6.9	<0.001	√	
Homogentisate[1]	4.0	5.0	<0.001	√	
4-Hydroxybenzaldehyde[1]	14.4	14.4	<0.001		√
N-Acetyl-L-tyrosine[1]	15.5	15.2	<0.001		√
γ-L-Glutamyl-L-tyrosine[1]	3.2	3.0	<0.001		√
3-(4-Hydroxyphenyl)lactic acid[1]	6.4	6.4	<0.001		√
L-Tyrosine[1]	2.7	2.7	<0.001		√
Mandelic acid[1]	12.6	12.0	<0.001		√
Inosine[3]	10.8	10.8	<0.001	√	
Uridine[7]	2.1	1.5	<0.001	√	

Table 3.1. Serum metabolite changes identified post-nitisinone therapy at 12 and 24 months in patients with AKU using an in-house AMRT

database. Abundance expressed as log_2 fold change (FC) compared to baseline (pre-nitisinone treatment). Log_2 FC included if >2 at 12 and or 24 months; p value <0.05 deemed significant. Metabolic pathway affected denoted by a number: 1 – tyrosine metabolism; 2 – tryptophan metabolism; 3 – purine metabolism; 4 – other metabolic processes; 6 – citric acid metabolism; 7 – pyrimidine metabolism.

Nine (43 %) of the 21 metabolites that were affected following treatment with nitisinone relate to tyrosine metabolism. Many of the other metabolites that had altered abundance following nitisinone therapy did not follow a clear theme apart from tryptophan, citric acid cycle and purine/pyrimidine metabolism.

3.5 Discussion

Over the last 2 decades there have been several reports on the use of nitisinone to treat AKU. Its inhibition of HPPD (Figure S3.1) has been shown to dramatically reduce the circulating concentration of HGA (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Ranganath *et al.,* 2018), but leads to marked hypertyrosinaemia (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016, Milan *et al.,* 2017; Ranganath *et al.,* 2018; Davison *et al*. 2018a; Davison *et al.,* 2018b; Davison *et al.,* 2018c). Beyond hypertyrosinaemia there is very little reported on the biochemical consequences of nitisinone therapy. Herein we report the impact of nitisinone therapy on the serum metabolome, using LC-QTOF-MS and a validated strategy to identify metabolites using an AMRT database developed in-house (Norman *et al.,* 2019a). This study is unique as it includes the largest cohort of patients with AKU to date, taking a 2 mg daily dose of nitisinone over 24 months. In addition, as it is based on the analysis of serum it is a better reflection of the impact of nitisinone on internal homeostasis.

This study confirms previous reports that nitisinone treatment in AKU results in a marked reduction in serum HGA and increase in tyrosine. In addition, a significant increase in 3-(4-hydroxyphenyl)lactic (HPLA) was observed. This marked increase in HPLA is also an expected consequence of nitisinone therapy (Gertsman *et al*., 2015a). Unexpectedly 3-(4-hydroxyphenyl)pyruvic acid (HPPA), the metabolite immediately proximal to the site of action of nitisinone, was not increased (Figure S3.1). There is an equilibrium between HPPA and HPLA (Figure S3.1) and it is possible that the reason for only observing a significant increase in HPLA is that the sample conditions (*e.g.* pH) shifted the equilibrium position to favour its formation. Moreover it is not known whether HPPA is a labile metabolite and thus may have degraded. A similar pattern where HPLA was the predominant metabolite has been previously reported (Norman *et al.,* 2019a), in a study that reported on the urine metabolome of patients with AKU treated with nitisinone; a 84 and 16 fold (raw FC) increase in HPLA and HPPA were reported, respectively.

Marked increases in the tyrosine conjugates N-acetyl-L-tyrosine and γ-glutamyl-tyrosine were also observed. The $\log_2$ FC observed was very similar for tyrosine and γ-glutamyl-tyrosine, but that of N-acetyl-L-tyrosine was markedly higher. The latter suggests that an equilibrium shift between tyrosine and N-acetyl-L-tyrosine may exist, but not for tyrosine and γ-glutamyl-tyrosine. Norman *et al.* (2019a) showed that urinary tyrosine was significantly higher than N-acetyl-L-tyrosine and γ-glutamyltyrosine in a cohort of AKU patients on 2 mg daily of nitisinone supporting that an equilibrium shift between tyrosine and tyrosine conjugates exists. In contrast, Gertsman *et al.* (2015a) reported a proportional increase in plasma tyrosine, N-acetyl-L-tyrosine and γ-glutamyl-tyrosine following nitisinone suggesting there is no equilibrium shift. While direct comparisons between the magnitudes of FC cannot be made as herein we report $\log_2$ FC, the proportions of metabolites are clearly different between the 2 studies. This may be due to the small number of patients in the study reported by Gertsman *et al.* (2015a) and that a 2 mg dose was used only in our study.

Nonetheless our findings support that non-traditional metabolic pathways are active in the face of tyrosine excess.

The elevated γ-glutamyl-tyrosine suggests that glutathione metabolism and the redox state of cell (Griffith *et al.,* 1979; Zhang *et al.,* 2009) are altered following treatment with nitisinone. This is of particular importance to AKU as elevated HGA is thought to lead to a pro-oxidant environment where 'soluble melanins' are formed (Davison *et al.,* 2016). Increasing evidence to support HGA induced oxidative stress has been reported *in vitro* in serum (Braconi *et al.,* 2011) and cellular models (Braconi *et al.,* 2010), and in patients with AKU (Millucci *et al.,* 2014). For a recent review on oxidative stress and its contribution to the mechanisms of the ochronotic process see Braconi *et al.* (2015). One may postulate that treatment with nitisinone reduces the burden on the glutathione cycle improving glutathione availability. In turn this may enable transfer of the glutamyl moiety from glutathione to tyrosine, via the action of γ-glutamyl-transpeptidase (Griffith *et al.,* 1979; Zhang *et al.,* 2009) to form γ-glutamyl-tyrosine. Interestingly, this study also revealed a marked decrease in the citric acid cycle metabolites α-ketoglutaric and succinic acid, the keto acids of α-ketoglutarate and succinate, following nitisinone. In this study glutamine and glutamatic acid did not change, thus one may hypothesise that glutamate (not measured) formed from glutamine is preferentially converted to glutathione and not α-ketoglutaric and succinic acid (Figure 3.2). The significance of this is not understood and requires further investigation.

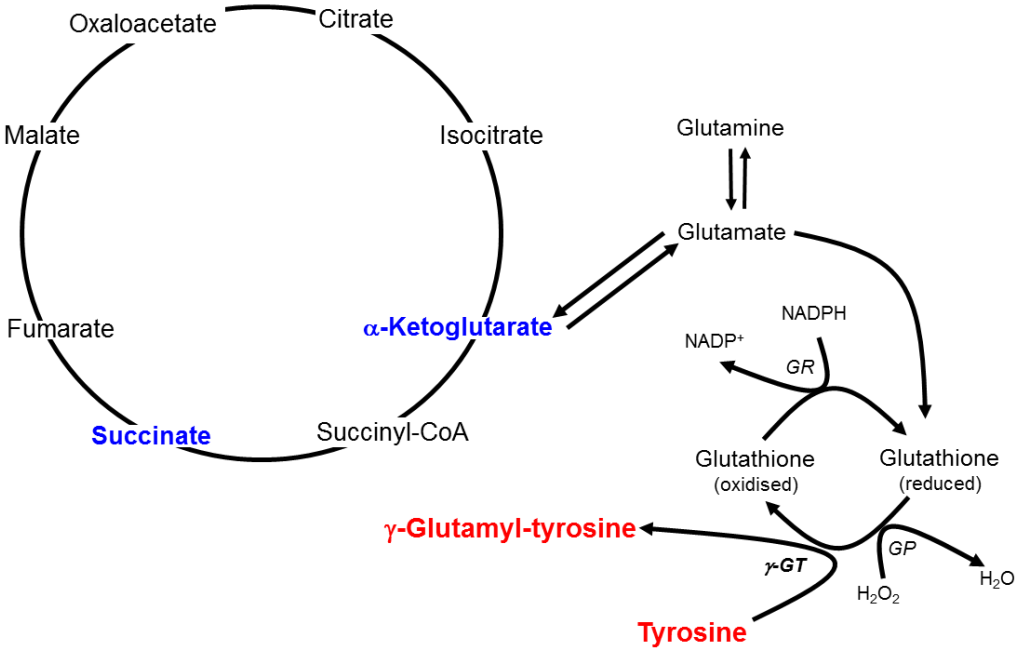

Figure 3.2. Proposed mechanism for the formation of γ-glutamyl-tyrosine in AKU following nitisinone therapy. Entities in red and blue represent an increase and decrease in metabolite abundance, respectively. GR – glutathione reductase; GP – glutathione peroxidase; GT – γ-glutamyltranspeptidase.

The significance of increased N-acetyl-L-tyrosine is unknown, but has been reported in serum (Gertsman *et al.,* 2015a) and urine (Norman *et al.,* 2019a) of AKU patients treated with nitisinone. It has also been observed in the urine of patients with hereditary tyrosinaemia type 2 (OMIM 276600) (Macsai *et al.,* 2001). One may postulate that N-acetyl-L-tyrosine represents a more efficient way to eliminate the excess tyrosine from the body as it is more water soluble than tyrosine. Interestingly N-acetyl-L-tyrosine has also been reported as an additive to foodstuffs given to patients on nutritional support (Hoffer *et al.,* 2003).

4-Hydroxyphenylacetic (HPA) and 4-hydroxybenzaldehyde (4-HBA) were increased and benzaldehyde decreased following treatment with nitisinone; these have not been previously reported in the serum metabolome of

patients with AKU following nitisinone treatment. HPA has been reported in urine following treatment with nitisinone (Norman *et al.,* 2019a). 4-HPA is generated from gut microbiota (Liu *et al.,* 2016); one may hypothesise that the increase observed herein resulted from less oxidative stress following nitisinone, which increased microbiotic metabolism. This is the first report in humans, but has been seen previously observed in a rat model of oxidative stress (Liu *et al.,* 2016).

The increase in HBA has been reported in the urine of patients with AKU following nitisinone (Norman *et al.,* 2019a), but the decrease in benzaldehyde has never been observed. The significance of both entities is uncertain. 4-HBA is a naturally occurring compound that originates from the saprophytic perennial *Gastrodia elata* (Kang *et al.,* 2017). Recent studies have reported the therapeutic effects of benzaldehydes in a number of areas including wound healing, cancer, vascular disease, and renal disease (Lee *et al.,* 2010; Moon *et al.,* 2012; Kong *et al*., 2014; Lee *et al.,* 2014). In the context of AKU the significance of 4-HBA and benzaldehyde are unknown and require further investigation. One may speculate that they relate to the benzoquinones, proposed as intermediates in the formation of ochronotic pigment observed in AKU (Norman *et al.,* 2019a).

Mandelic acid was also increased following treatment with nitisinone, which has been reported previously (Norman *et al.,* 2019a). The significance of this is uncertain, but urinary elevation has previously been observed in patients with PKU on a phenylalanine restricted diet (Rampini *et al.,* 1974).

The ‘off target effects’ of any drug are essential when considering its suitability in treating a patient (Lynch *et al.,* 2017). Nitisinone therapy in AKU and HT1 have long been associated with altered tyrosine metabolism, and so can be considered a ‘targeted effect’. More recently off targets effects of nitisinone have been reported due to its impact on tryptophan metabolism. A decrease in 5-hydroxyindoleacetic acid (serotonin metabolite) has been reported in the cerebrospinal fluid and urine of patients with HT1 (Thimm *et al.,* 2011) and AKU (Davison *et al.,* 2018b; Davison *et al.,* 2018c),

respectively. Serum tryptophan itself has been shown not to change following treatment with nitisinone (Davison *et al.,* 2018a); in contrast urinary tryptophan has been shown to decrease (Norman *et al.,* 2019a). These differences may be explained by the fact that tryptophan is highly protein bound (~90-95 %) (Cervenka *et al.,* 2017) and measurement in serum reflects total tryptophan and urinary tryptophan reflects free tryptophan. The biologically active fraction is the free fraction, which is not typically measured in serum, and the majority is metabolized via the kynurenine pathway (Cervenka *et al.,* 2017). Herein I-3-L, 4-quinolinecarboxylic acid and trigonelline were the only tryptophan-related metabolites that were changed following nitisinone. The significance of the latter 2 metabolites is uncertain, but reinforces that downstream metabolism of kynurenine and niacin may be altered following nitisinone treatment. Gertsman *et al.* (2015b) also demonstrated an increase in plasma I-3-L, but additionally indole-3-pyruvate (I-3-P). It has been proposed that the increased activity in the indole pyruvate pathway results from tryptophan aminotransferase having a higher affinity for tryptophan in the presence of keto acids (*e.g.* HPPA) (Lees *et al.,* 1973). The reason for not observing I-3-P is uncertain, however one may speculate that I-3-L and not I-3-P was increased due to reaction conditions favoring its formation. In addition, differences may in part be explained by different approaches to sample preparation, chromatographic and mass spectrometry conditions, patient cohorts and nitisinone doses used. In addition a separate study showed urinary xanthurenic acid, and L-kynurenine and indoxyl-sulfate were increased and decreased following nitisinone therapy, respectively (Norman *et al.,* 2019a) The significance of these changes are unknown.

Beyond tryptophan metabolism there are limited reports on the off-target effects of nitisinone on the metabolome. Herein we have shown that trans-4-hydroxyproline decreases significantly following treatment. This is of particular relevance to AKU as it suggests that there may be decreased collagen breakdown. This requires further investigation as previous authors (Taylor *et al.,* 2017) have demonstrated a very low cartilage turnover state in AKU patients. In contrast, a different study reported that cartilage

degradation, as well as bone resorption markers were elevated in AKU patients compared to controls (Genovese *et al.,* 2015).

In addition, significant decreases were observed in the purine and pyrimidine precursor's inosine and uridine, respectively. In a previous study decreased excretion of the purine metabolites adenine and allantoin were reported in urine from AKU patients and an AKU mouse treated with nitisinone (Norman *et al.,* 2019a). Patients included in this study and previous (Norman *et al.,* 2019a) were not on uric acid-lowering medication, however were on a protein-restricted diet which may contribute to this change. A plausible explanation for the observations in serum and in urine may also relate to changes in the oxidative state of the cell. Allantoin has previously been suggested as a marker of oxidative stress (Marrocco *et al.,* 2017), one may postulate that the decreases observed in purine and pyrimidine metabolites are a reflection of reduced oxidative stress in the face of lower HGA concentrations (Marrocco *et al.,* 2017).

Four unrelated entities were also shown to be decreased following nitisinone therapy; glycocholate, homoserine, mevalolactone and allothreonine. The reasons for this remain of unknown significance and requires further investigation.

It is important to highlight several limitations in this study. Firstly, while the number of patients included in this study is the largest reported to date, metabolites identified need validating in a larger cohort of patients. Moreover, an untreated group of AKU patients would provide greater credibility that the findings presented herein are a consequence of nitisinone therapy. In addition, while we believe the AMRT database used in this study is comprehensive, the targeted evaluation of the serum metabolome precludes the identification of novel changes in metabolites. Furthermore, the use of non-selective sample preparation to gain broad coverage of the metabolome may have limited metabolite detection if present at a low concentration or if changes were not reproducible due to ion suppression.

3.6 Conclusions

Evaluation of the impact of nitisinone treatment on the serum metabolome of patients with AKU revealed a number of novel changes including a number that are not directly related to the tyrosine metabolic pathway. Some of these changes can be explained by the impact of nitisinone therapy on the cellular redox state and the wider impact of metabolites on enzyme activity. Further work is required to provide greater insight into the changes observed in the serum metabolome following nitisinone therapy.

3.7 Supplementary material

3.7.1 Quadrupole time of flight mass spectrometer operating conditions

Capillary and fragmentor voltages were 4000 V and 380 V, respectively. Desolvation gas temperature was 200 °C with flow rate at 15 L/min. The sheath gas temperature was 300 °C with flow rate at 12 L/min and nebulizer pressure was 40 psi and nozzle voltage 1000 V. Data acquisition rate was 3 spectra/s.

3.7.2 Preparation of reference mass correction solution

Reference mass correction solution was prepared in 95:5 methanol:water containing 5 mmol/L purine (CAS No. 120-73-0), 100 mmol/L trifluoroacetic acid ammonium salt (TFA, CAS No. 3336-58-1) and 2.5 mmol/L hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine (HP-0921, CAS No. 58943-98-9) (Agilent, Cheadle, UK).

Reference ions monitored were: purine (*m/z* 121.0509) and HP-0921 (*m/z* 922.0098) (positive polarity) and TFA (*m/z* 112.9856), purine (*m/z* 119.0363) and HP-0921 (HP-0921 + formate adduct: *m/z* 966.0007) (negative polarity).

3.7.3 Data acquisition and handling parameters

Data were acquired using Acquisition (Build 06.00, Agilent, Cheadle, UK). Quality checks and processing of raw data files (Agilent '.d' files) were performed with Qualitative Analysis software (Build 07.00, Agilent, Cheadle, UK).

Extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run and that the reference ion signal did not drop out during the chromatographic run. In addition, to check chromatographic reproducibility binary pump pressure curves for injections across each analytical sequence were overlaid. Mass accuracy and chromatographic reproducibility were acceptable for all experiments performed.

Profiling sample data acquired were mined for signals representing AMRT database compounds using 'targeted feature extraction' with Profinder software (Build 08.00, Agilent, Cheadle, UK). Targeted feature extraction uses the molecular formulae from the AMRT database to extract and group spectral signals (*i.e.* adducts, isotopes and multimers) that correspond to individual database compounds. Feature extraction employed a window of theoretical accurate mass ±10 ppm and database retention time ±0.3 min. Allowed species were: H^+, Na^+ and NH_4^+ (positive polarity) and H^- and CHO_2^- (negative polarity). Dimers were allowed for both polarities. Charge state range was 1-2.

3.7.4 Data quality control and statistical analysis

Quality control filters were applied to entities obtained from targeted feature extraction in each profiling experiment. Entities were retained if present in at least 2 samples per experimental group. Data files were then exported from Profinder as '.CEF' files as a whole batch for each profiling experiment and imported to Mass Profiler Professional (MPP) software (Build 14.5, Agilent, Cheadle, UK).

In MPP sample data files were scaled to the median of all samples and chemical entities were filtered based on the data from injection of QC pooled samples. Entities were first filtered based on frequency, and retained if observed in 100 % of replicate injections for at least 1 sample group pool. Further filtering was then based on variability, and entities were retained if peak area coefficient of variation remained <25 % across replicate injections for each sample group pool.

Statistical analyses performed in MPP were based on compound signal intensity, expressed as total peak area. Profiles were compared at 12 and 24 months on 2 mg daily nitisinone with pre-nitisinone profiles by 1-way repeated measures ANOVA with Benjamini-Hochberg false-discovery rate adjustment. Log_2 FC was calculated pairwise between all groups within each dataset, and this was based on raw peak area data. Changes were deemed significant if asymptotic p value was <0.05. Principal component analysis

employing a 4 component model were also performed on each filtered dataset using MPP.

3.7.5 Supplementary Figures

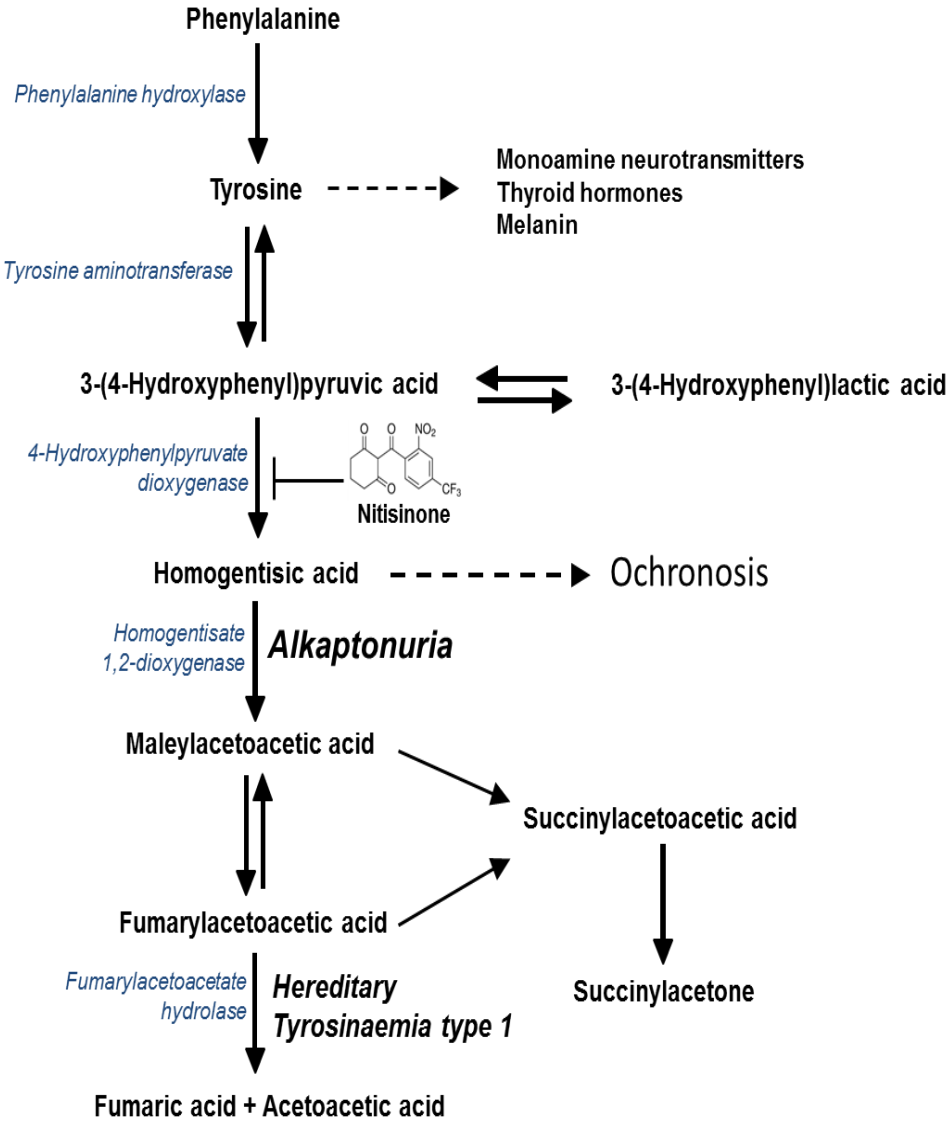

Figure S3.1.Tyrosine metabolic pathway. Highlighted are: (i) the metabolic fate of tyrosine in health, (ii) site of the enzyme defect observed in AKU, homogentisate 1,2-dioxygenase (HGD, EC 1.13.11.5) and HT1, fumarylacetoacetate hydrolase (FAH, EC 3.7.1.2), and (iii) the site where nitisinone inhibits 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.13.11.27) activity.

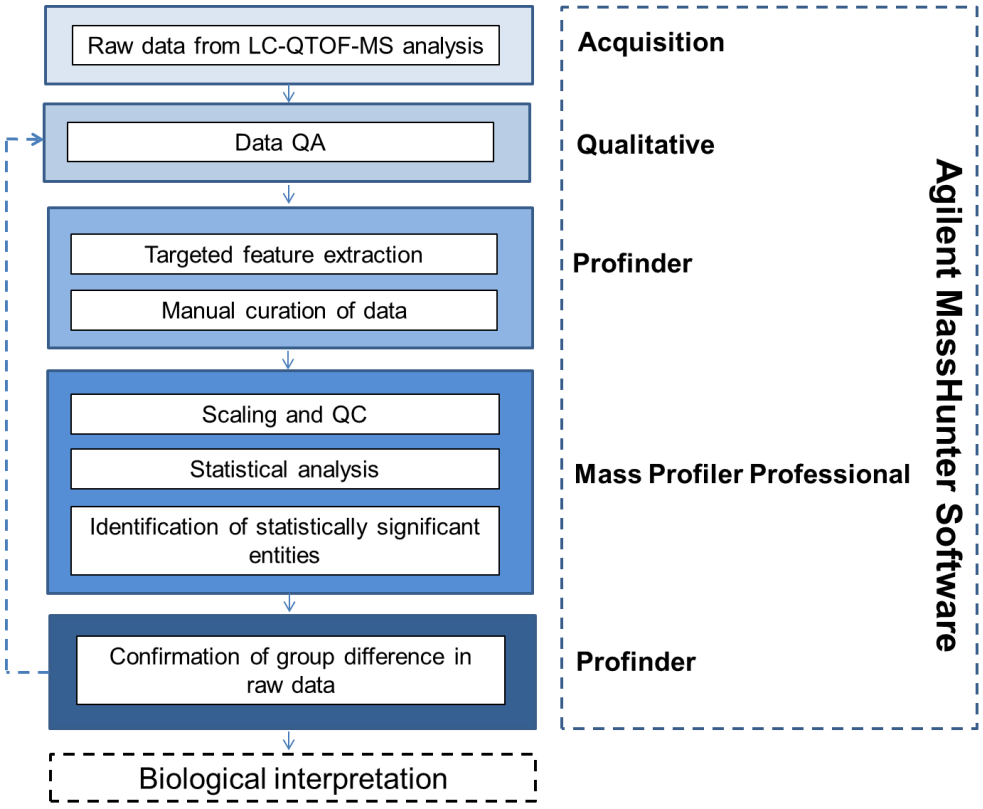

Figure S3.2. Work flow for processing of raw data from LC-QTOF-MS analysis using the Agilent MassHunter software suite (Agilent, Cheadle, UK). QA – quality assessment; QC – quality control.

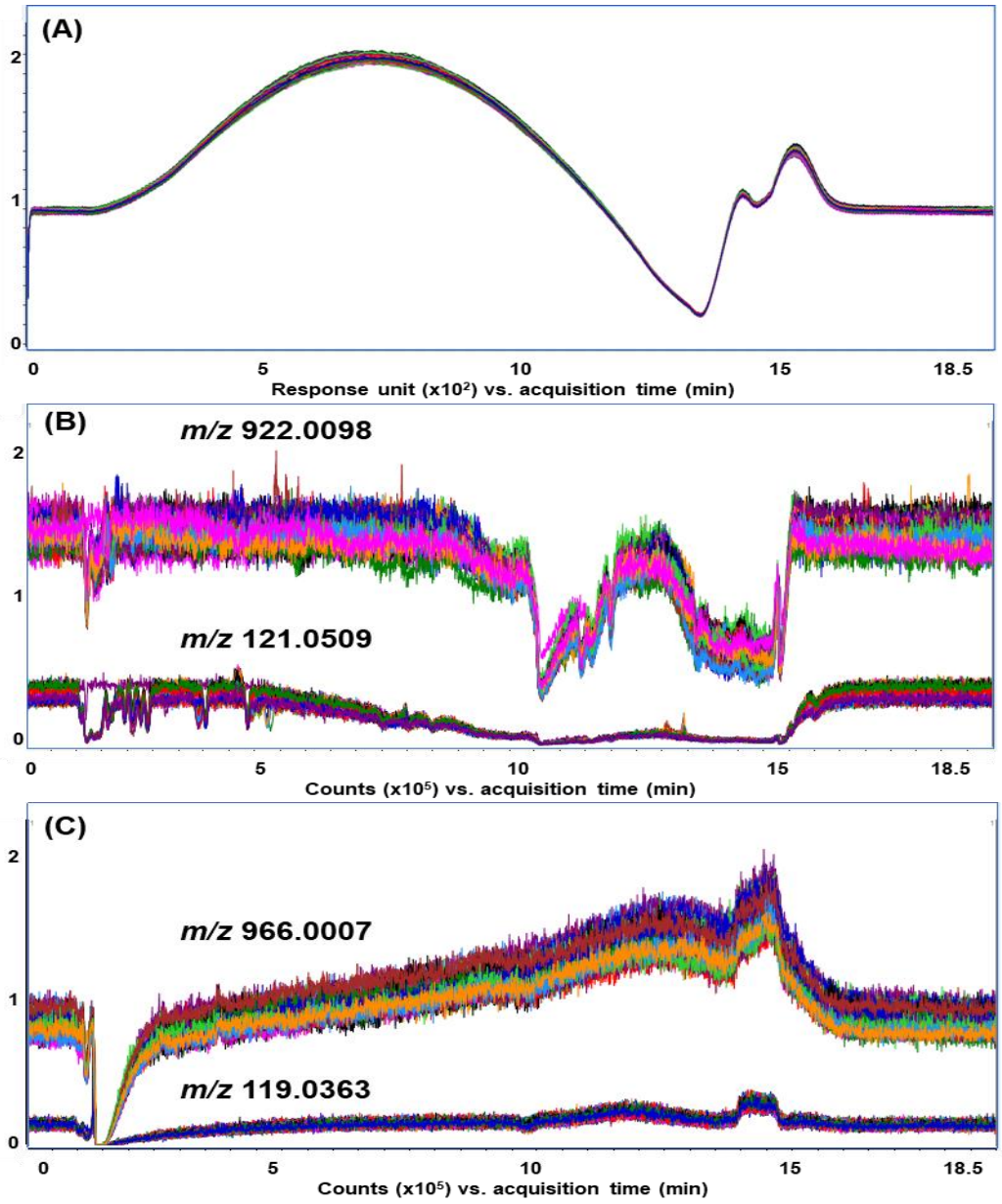

Figure S3.3. Representative data from quality checks performed on data acquired from LC-QTOF-MS analysis of serum samples in positive and negative polarities (each analytical sequence consisted of 125 samples). (A) Overlaid binary pump pressure curves, (B) Overlaid reference ion signal in positive polarity and (C) Overlaid reference ion signal in negative polarity, extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run. Reference ion signal is present during the analytical runs in both polarities, in negative polarity the greatest suppression of signal occurred at 1.5 min. Ion suppression in this region is greatest due to elution of poorly retained entities.

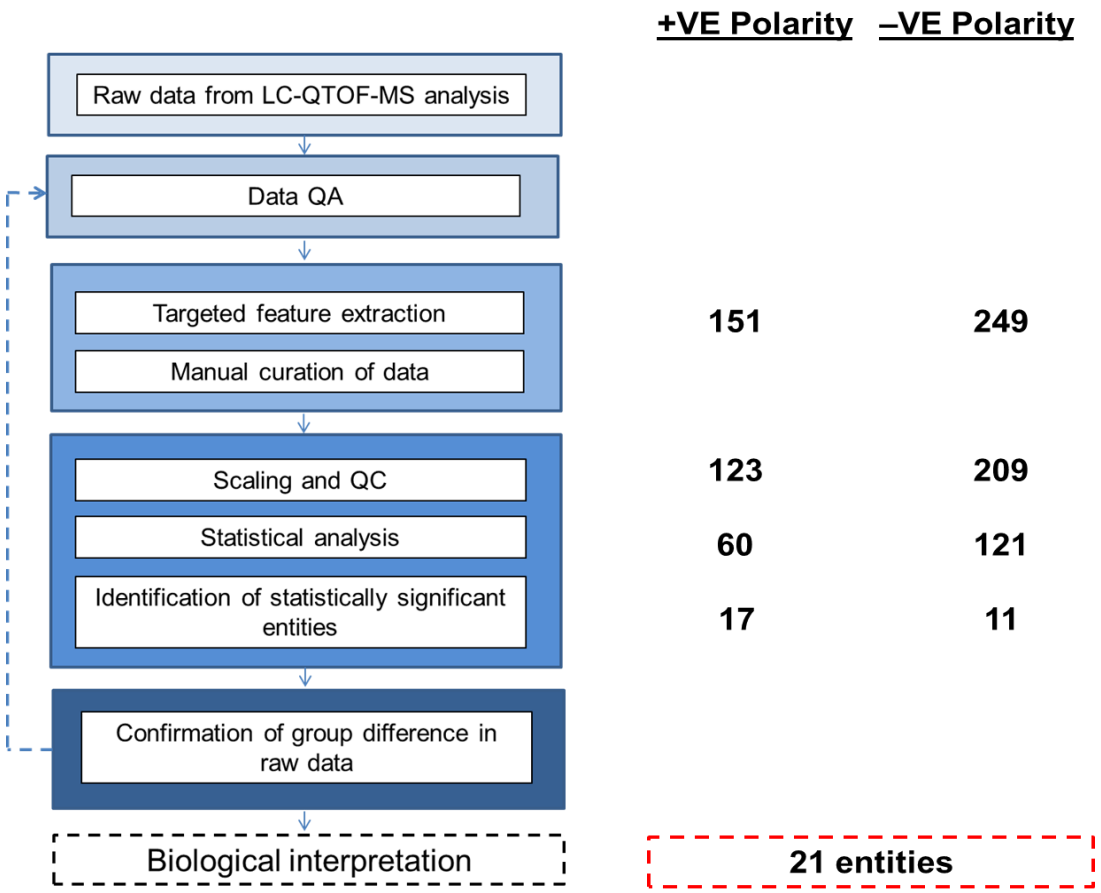

Figure S3.4. Number of accurate mass retention time (AMRT) matches obtained from processing raw LC-QTOF-MS data. AMRT matches obtained using the batch targeted feature extraction algorithm (Build 08.00, Agilent, Cheadle, UK), and subsequent entities retained following quality control filtering and statistical analysis in positive (+VE) and negative (-VE) polarity; QA – quality assessment; QC – quality control.

3.7.6 Supplementary Tables

Metabolite name	Empirical formula	Mass	Retention time (min)
N-Acetyl-L-tyrosine	C9H11NO3	223.0844	4.81
γ-Glutamyl-tyrosine	***C14H18N2O6***	***310.1164***	**3.81**
Indole-3-lactate	***C11H11NO3***	***205.0738***	**6.66**

Table S3.1. Summary of accurate mass and retention time data for metabolites that make up in-house AMRT database used in this study. These can be found at https://doi.org/10.6084/m9.figshare.c.4378235.v2. This study adapted this library to include the additional metabolites below. The accurate mass and retention time of compounds in ***bold italics*** are based on elution time associated with theoretical monoisotopic mass and were not verified with an analytical standard

Compound matched to AMRT database	Polarity	
	Positive	Negative
(R)-Malate	x	X
(S)-Malate	x	X
1,2-Didecanoyl-Sn-glycero-3-phosphocholine		X
1-Aminocyclopropane-1-carboxylate	x	x
1-Methyladenosine	x	x
1-Methylnicotinamide		x
2-Amino-2-methylpropanoate		x
2-Deoxy-D-glucose	x	x
2-Deoxyuridine 5-mono-phosphate		x
2-Hydroxybutyric acid		x
2-Methylglutaric acid	x	
2-Quinolinecarboxylic acid	x	
3-(4-Hydroxyphenyl)lactic acid		x
3,4-Dihydroxyphenylacetate	x	
3-Amino-4-hydroxybenzoic acid		x
3-Amino-5-hydroxybenzoic acid		x
3-Aminoisobutanoate		x
3-Dehydroshikimate		x
3-Hydroxykynurenine		x
3-Methoxy-L-tyrosine	x	x
3-Methoxytyramine	x	x
3-Methyglutaric acid	x	
3-Methyladenine		x
3-Methylhistamine		x
3-Sulfino-L-alanine		x
3-Ureidopropionate	x	x
3α,12α-Dihydroxy-5β-cholanate	x	x
4-Aminobutanoate		x
4-Hydroxy-2-quinolinecarboxylic acid	x	
4-Hydroxy-3-methoxyphenylglycol		x
4-Hydroxybenzaldehyde	x	
4-Hydroxy-L-proline	x	x
4-Hydroxyphenylacetic acid		x
4-Imidazoleacetic acid		x
4-Methyl-2-oxo-pentanoic acid		x
4-Pyridoxate	x	x
4-Quinolinecarboxylic acid		x
5-Aminolevulinic acid	x	x
5-Hydroxyindoleacetate		x
5-Hydroxy-L-tryptophan	x	x

5-Methylthioadenosine	x	x
5-Oxo-D-proline	x	x
5-Oxo-L-proline	x	x
6-Carboxyhexanoate	x	
6-Deoxy-L-galactose	x	x
N-acetyl-L-tyrosine	x	
Adenine		x
Adenine hydrochloride hydrate		x
Adenosine 5-monophosphate		x
Agmatine sulfate		x
Allantoin		x
Allose	x	x
Arabinose		x
Azelaic acid	x	x
Azelaic acid		x
Benzaldehyde		x
Biliverdin		x
Bis(2-ethylhexyl)phthalate	x	x
Caffeine	x	x
Carnosine		x
Chenodeoxycholate		x
Cholesteryl acetate	x	x
Cis-4-hydroxy-D-proline	x	x
Citrate	x	
Citrulline	x	x
Corticosterone		x
Cortisol	x	x
Cortisol 21-acetate		x
Creatine	x	x
Creatinine	x	x
Cytidine		x
Cytidine 5-diphosphocholine		x
D-(-)-Arabinose		x
D-(+)-Cellobiose		x
D-(+)-Galactosamine	x	x
D-(+)-Galacturonic acid	x	x
D-(+)-Glucosamine	x	x
D-(+)-Trehalose		x
D-Alanine		x
D-Aspartate		x
Dehydroascorbate	x	
Deoxycarnitine	x	
Deoxycarnitine		x

Deoxycholate		x
Deoxycytidine		x
D-Fructose-6-phosphate	x	x
D-Galactose	x	x
D-Gluconate		x
D-Glucono-1,5-lactone		x
D-Glucosamine-6-phosphate		x
D-Glucose-6-phosphate	x	x
D-Glucuronic acid	x	x
D-Glucuronolactone	x	x
D-Glyceric acid		x
D-Gulonic acid-γ-lactone		x
Diacetyl	x	x
Diethanolamine		x
D-Lactose		x
DL-Kyneurenine	x	x
DL-Normetanephrine		x
D-Lyxose		x
D-Mannosamine	x	x
D-Mannose 6-phosphate	x	x
Docosahexaenoic acid		x
D-Ornithine	x	x
D-Pantothenic acid	x	x
D-Psicose	x	x
D-Ribose		x
D-Ribose-5-phosphate		x
D-Sorbitol	x	x
D-Tagatose	x	x
D-Tryptophan	x	x
D-Xylose		x
Adrenaline	x	x
Erythritol	x	x
Ethylmalonic acid		x
Galactitol	x	x
γ–Glutamyl-tyrosine*	x	
γ–Linoleic acid		x
Gluconic acid		x
Glucosaminate		x
Glyceraldehyde	x	x
Glycerol	x	x
Glycerol 2-phosphate	x	x
Glyceryl trimyristate		x
Glycine	x	x

Glycocholate	x	x
Guanidinoacetate		x
Guanine	x	x
Guanosine		x
Hexadecanol		x
Hippurate	x	x
Homocysteine		x
Homocystine	x	x
Homogentisate		x
Homoserine	x	x
Homovanillate	x	x
Hypotaurine	x	x
Hypoxanthine	x	x
Indole-3-acetaldehyde		x
Indole-3-acetate	x	x
Indole-3-acetic acid	x	x
Indole-3-lactate*	x	
Indole-3-pyruvic acid		x
Inosine	x	x
Isocitric acid	x	x
L-Alanine		x
L-Allothreonine	x	x
L-Arabitol	x	x
L-Arginine	x	x
L-Asparagine	x	x
L-Aspartate		x
Lauric Acid		x
Lauroylcarnitine		x
L-Carnitine	x	x
L-Cystathionine	x	
L-Cystine	x	x
Leucine	x	
L-Glutamic acid	x	x
L-Glutamine	x	x
L-Histidine	x	x
Linoleate		x
L-Isoleucine	x	x
L-Kynurenine	x	x
L-Lysine	x	x
L-Lysine monohydrochloride	x	x
L-Methionine	x	x
L-Norvaline	x	x
L-Ornithine	x	x

L-Phenylalanine	x	
L-Pipecolic acid	x	x
L-Proline	x	x
L-Rhamnose	x	x
L-Serine	x	x
L-Sorbose	x	x
L-Threonine	x	x
L-Tryptophan	x	x
L-Tyrosine	x	x
L-Valine	x	x
Maleamate		x
Maleic acid	x	
Maltose		x
Mandelic acid	x	x
Mannitol	x	x
Mannose	x	x
Melibiose		x
Methyl jasmonate	x	
Methyl β-D-galactoside		x
Methylmalonate		x
Mevalolactone		x
Myo-inositol	x	x
N-(Pai)-Methyl-L-histidine	x	x
N-Acetyl-D-galactosamine		x
N-Acetyl-D-glucosamine		x
N-Acetyl-DL-glutamic acid	x	x
N-Acetyl-DL-methionine	x	x
N-Acetyl-DL-serine	x	x
N-Acetyl-D-mannosamine		x
N-Acetyl-D-tryptophan		x
N-Acetylglycine	x	x
N-Acetyl-L-alanine	x	x
N-Acetyl-L-aspartic acid	x	x
N-Acetylneuraminate	x	x
N-Acetylputrescine		x
N-Acetylserotonin		x
N-Amidino-L-aspartate		x
Nicotinamide	x	x
Nicotinamide mononucleotide		x
N-Methyl-D-aspartic acid	x	x
N-Methyl-L-glutamate		x
Norleucine	x	
N-α-Acetyl-L-asparagine		x

Nε,Nε,Nε-Trimethyllysine		x
O-Acetyl-L-serine	x	x
Octopamine		x
Orotate		x
Orotic acid		x
Palatinose		x
Palmitate		x
Palmitoleic acid		x
Paraxanthine	x	x
Phenethylamine		x
Phenylacetaldehyde		x
Phenylacetic acid		x
Phenylethanolamine		x
Phosphocholine	x	
Phylloquinone		x
Pipecolate	x	x
Pyridoxal	x	x
Pyridoxamine	x	x
Quinate		x
Reichstein substance S		x
Ribitol	x	x
Riboflavin		x
S-(5-Adenosyl)-L-methionine		x
Sarcosine		x
Serotonin		x
Shikimate		x
S-n-Glycerol 3-phosphate	x	x
Sorbate		x
Spermidine	x	x
Sphinganine		x
Sphingomyelin	x	
Suberic acid	x	x
Succinic acid		x
Sucrose		x
Taurine	x	x
Theobromine	x	x
Theophylline	x	x
Thiamine monophosphate		x
Thymidine		x
Thymine		x
Trans-4-hydroxyproline	x	x
Trans-aconitate		x
Trigonelline	x	x

Uracil	x	x
Urate	x	x
Uridine	x	x
Urocanate	x	x
Xanthine	x	x
Xanthosine		x
Xanthurenic acid		x
Xylitol	x	x
α-Aminoadipate	x	x
α-D-Galactose 1-phosphate	x	x
α-D-Glucose	x	x
α-D-Glucose 1-phosphate	x	x
α-Ketoglutaric acid	x	x
β-Alanine		x
Bine	x	x
Ω-Hydroxydodecanoic acid		x

Table S3.2. Summary of metabolites that were aligned and matched across all samples at baseline, and after nitisinone treatment at 12 and 24 months. Metabolites were aligned using Profinder software (Build 08.00, Agilent, Cheadle, UK); a targeted feature extraction was used to align profiled experimental data with data in the accurate mass and retention time (AMRT) database containing 469 intermediary metabolites. Metabolites aligned and matched in both negative (n=249) and positive (n=151) polarities are detailed. Feature extraction employed a window of theoretical accurate mass ± 10 ppm and retention time ± 0.30 min. Allowed species were: H^+, Na^+ and NH_4^+ for positive polarity; and H^- and CHO_2^- for negative polarity. Dimers were allowed for both polarities. Charge state range was 1-2. * – accurate mass and retention time of compounds are based on elution time associated with theoretical monoisotopic mass.

Appendix 1

Evaluation of the serum metabolome of patients with alkaptonuria after 2 years of treatment with nitisinone using 1D Proton (^{1}H) nuclear magnetic resonance

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Unpublished.

3A.1 Introduction

Background on Alkaptonuria (AKU) and the rationale for evaluating the serum metabolome following treatment with nitisinone in patients with AKU is not presented herein as this is comprehensively covered in the introduction of Chapter 3. This study differs only in that the analytical technique used for sample analysis was 1D ^{1}H nuclear magnetic resonance (NMR).

Increasingly it is recognized that a metabolomic study is defined by the capability and limitations of the analytical methodologies employed, therefore the use of a single analytical methodology may hinder this and provide an incomplete biological picture (Marshall *et al.,* 2017). As NMR and mass spectrometry based methodologies are complementary their combination is likely to improve the overall quality of a study and enhance the coverage of the metabolome, moreover it is more likely that the structural elucidation of unknown metabolites is possible (Marshall *et al.,* 2017). It is for this reason NMR analysis was performed in addition to LC-QTOF-MS analysis (Chapter 3).

3A.2 Materials and methods

3A.2.1 Patients and serum sample collection

Serum samples (S-monovette, Sarstedt, Germany) used were those that were collected from AKU patients that provided samples for LC-QTOF-MS analysis (see Chapter 3). Following centrifugation of samples at 1500 ×*g* for 10 min at 4 °C, and prior to acidification (see section 3.3.2.2) an aliquot of serum (non-acidified) was made and stored at -20 °C until analysis.

For demographic details of the patients included in this analysis see Chapter 3. In brief, paired samples from 25 patients were analysed from baseline (pre-nitisinone treatment) and after 12 and 24 months of treatment with nitisinone.

3A.2.2 NMR methodology

3A.2.2.1 Sample preparation for ^{1}H NMR

Stored serum aliquots were thawed immediately prior to NMR sample preparation. 100 μL of each serum sample had 100 μL of deuterated buffer (200 mmol/L sodium phosphate buffer pH 7.4 and 100 mmol/L sodium azide) added to it and was vortexed for 20 s and centrifuged at 12,000 *g* for 1 min at 4 °C. 180 μL of the sample mixture then was transferred to 3 mm (outer diameter) NMR tubes (Bruker, UK) for acquisition.

3A.2.2.2 NMR acquisition and spectral processing

1D proton Carr-Purcell-Meiboom-Gill (1D ^{1}H CPMG, vendor supplied cpmgpr1d) NMR spectra were acquired at 700 MHz using a Bruker Avance III HD 700 MHz NMR spectrometer fitted with a 5 mm triple resonance cryoprobe and a chilled sample jet auto-sampler. Samples were referenced to anomeric ^{1}H of glucose at 5.24 ppm. ^{1}H NMR spectra had spectral widths of 17.16 ppm, 4 s relaxation delay, 32 scans with 4 dummy scans collected into 73 k data points (3.067 s acquisition time). Spectral acquisition was automated via iconNMR (Bruker, UK) following calibration of the spectrometer temperature, which was calibrated to 37 °C (± 0.1 °C) via a methanol thermometer (Ammann *et al.,* 1982). 3D shimming was also performed on a quality control sample of sucrose, and the spectrometer optimised to offset for water suppression on a single representative serum sample. All spectra were zero filled to 128 k data points with exponential line broadening of 0.3 Hz applied before fourier transformation. Spectra were automatically phased, referenced and baseline corrected in Topspin using vendor supplied automated routines (Version 3.5, Bruker, UK).

3A.2.2.3 Spectral data quality control, metabolite annotation and identification

Figure 3A.1 summarises the main steps taken after raw data were acquired. Serum metabolites in the ^{1}H NMR spectra were examined in Topspin by overlaying all spectra to assess (i) the shape and width of the glucose reference peak (5.24 ppm, line width measured at half peak height) (ii) that the baseline was flat without curvature or sine wiggle, (iii) that signal to noise

was as expected compared to a representative spectrum, (iv) that water suppression was good (*i.e.* narrow water signal between 0.2 and 0.4 ppm wide and no baseline distortion beyond this range and (v) phasing was good throughout spectra as recommended best practice in the metabolomics community (Sumner *et al.,* 2007; Considine *et al.,* 2019).

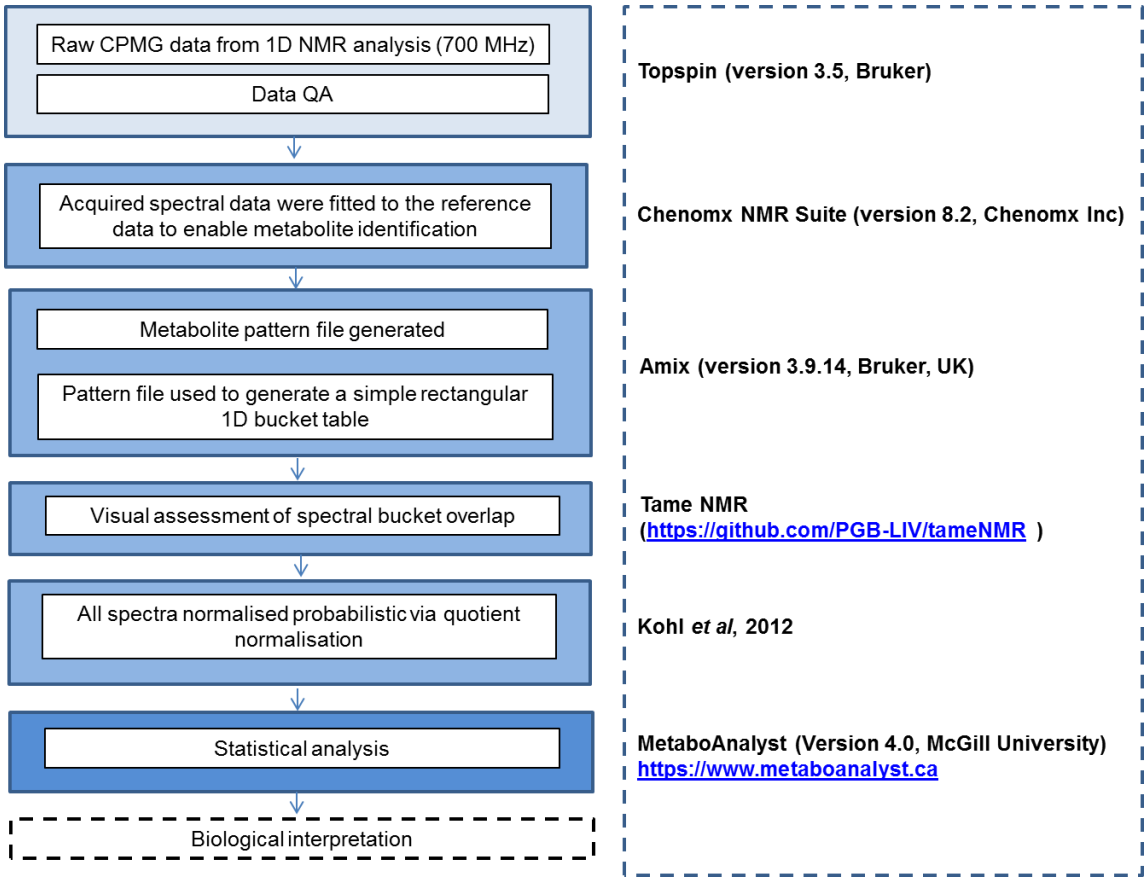

Figure 3A.1. Summary of data handling and statistical analysis performed following serum sample analysis with 1D ^{1}H NMR. QA – quality assessment.

Spectra were annotated using Chenomx (Version 8.2, Canada) by importing 2 representative spectra from the different treatment groups (pre- and post-nitisinone) into Chenomx. Acquired spectral data were fitted to the reference data to enable metabolite identification (Chenomx contains 328 metabolites). Identification enabled metabolite peak shift boundaries to be established.

These were then used to produce a pattern file. Metabolite peaks without an identity were labelled as ‘unknown’. The pattern file was used to generate a variable width 1D bucket table using AMIX (Version 3.9.14, Bruker, UK). Spectra were bucketed according to spectral features or peaks; all peaks both annotated and unknown were included in the bucket table.

Spectra were subjected to a quality control filter prior to statistical analysis by characterization of the glucose reference peak with spectra excluded that did not satisfy line width or baseline requirements in the spectral bucketing software AMIX.

To ensure spectral buckets did not overlap small windows from the entire spectrum were reviewed visually using tameNMR (https://github.com/PGB-LIV/tameNMR). TameNMR does this by superimposing the bucket table boundaries onto NMR spectra, 8 representative NMR spectra were used (pre- and post-nitisinone treatment).

Spectra were prepared for statistical analysis by normalizing each spectrum using the probabilistic quotient normalisation (PQN) method (Kohl *et al.,* 2012). The bucket table was imported into MetaboAnalyst (Version 4.0, https://www.metaboanalyst.ca) for statistical analysis in the format of a .csv file as a peak intensity table. No data filtering was applied to the data set as it contained <5000 features. Data were log transformed, and underwent Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). Statistical analysis included principal component analysis (PCA), and a 1-way ANOVA which was used to assess significant differences in metabolite patterns observed at 12 and 24 months compared to baseline following treatment with nitisinone. An adjusted p-value (Benjamini Hochberg, false discovery rate corrected) cutoff of 0.05 and a $\log_2$ fold change (FC) >2 were deemed significant.

3A.3 Results

Following NMR spectral data quality checks, 6 out of 75 samples were excluded from the data analysis. In total, 23/25 patients were included at baseline; 21/25 patients at 12 months and 24/25 patients at 24 months. All spectra included were manually aligned to glucose (5.24 ppm, see Figure 3A.2 for an example) before any statistical analysis. Figure 3A.3 shows representative aligned NMR spectra from a patient pre-nitisinone, and at 12 and 24 months after 2 mg daily nitisinone treatment. The most visible changes observed between the spectra are in the aromatic region highlighted by the dotted box.

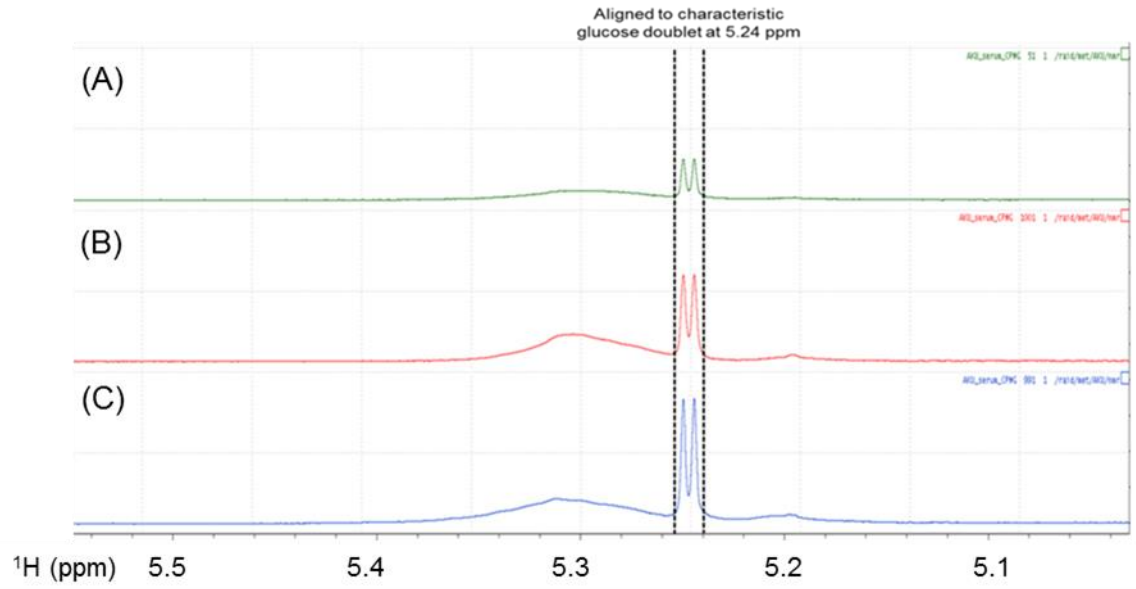

Figure 3A.2. NMR spectra showing spectral alignment to glucose at 5.24 ppm. Spectra from the analysis of serum samples are depicted (A) AKU patient pre-nitisinone, (B) AKU patient after 12 months of nitisinone treatment and (C) AKU patient after 24 months nitisinone treatment.

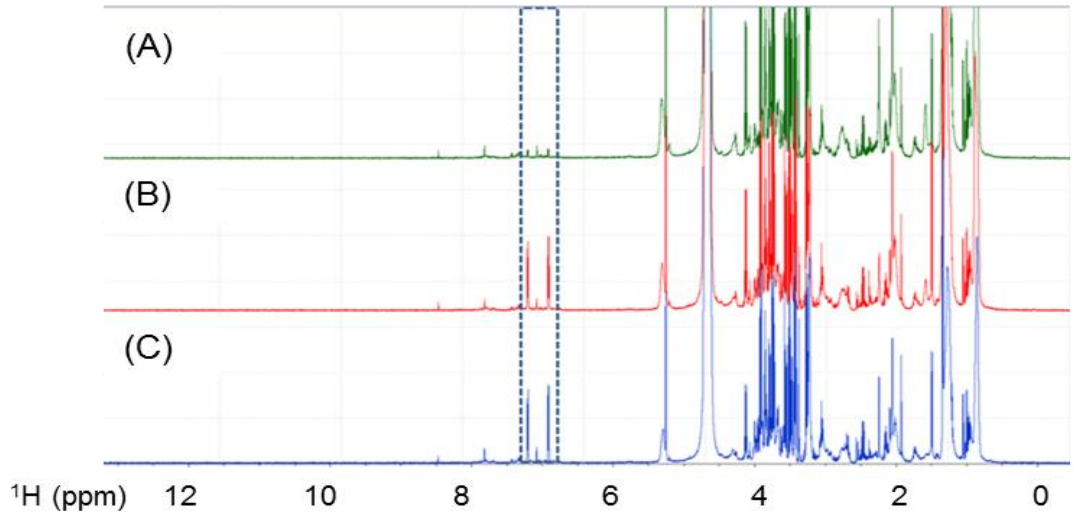

Figure 3A.3. Representative 1D ^{1}H NMR spectra from the analysis of serum taken from a patient. (A) Pre-nitisinone, (B) 12 months after nitisinone treatment and (C) 24 months after nitisinone treatment. The largest changes observed are in the aromatic region of the spectra highlighted by the dotted box.

In order to determine statistical and FC differences in NMR serum metabolite profiles a pattern file was made (see Supplementary material – Appendix Supplementary Table 3.1 for information contained in the pattern file) using representative NMR spectra (8 serum samples – 4 pre-nitisinone and 4 post-nitisinone). In total 119 spectral shifts were included in the pattern file, this corresponded to 37 metabolites matched in Chenomx, 15/37 metabolites had multiple spectral shifts. In addition there were 27 unknown chemical shifts, which relate to a maximum of 27 metabolites assuming 1 spectral shift relates to 1 metabolite. Figure 3A.4 shows the images generated using tameNMR to check spectral boundaries did not overlap in the pattern file.

All spectra generated from the analysis of all serum samples included in this study were aligned with the pattern file to generate a bucket table containing metabolite signal intensities for statistical analysis. PCA and associated loads (Figure 3A.5) show clear separation between samples collected at baseline, and at 12 and 24 months.

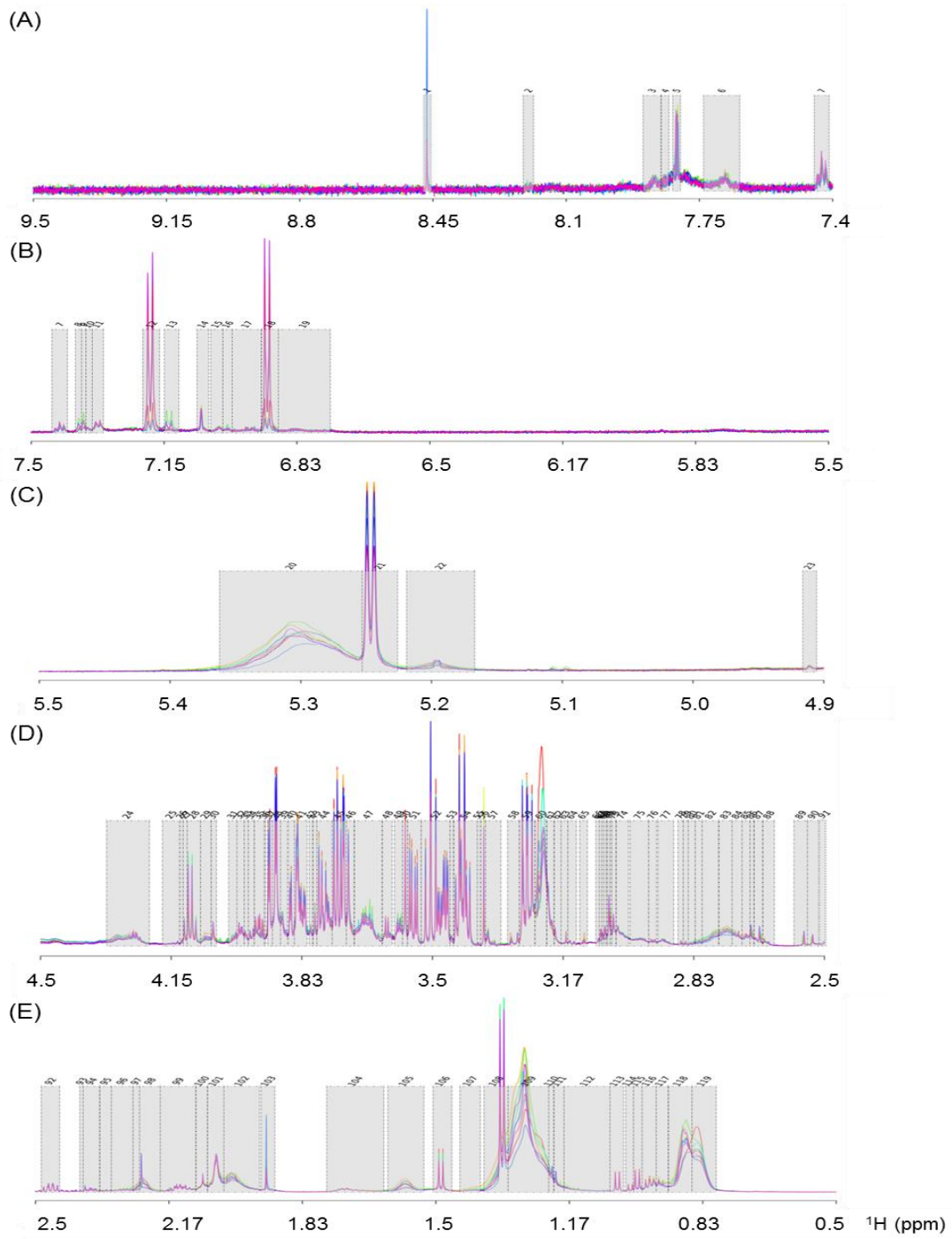

Figure 3A.4. TameNMR screen shots showing spectral boundaries used for individual metabolite spectral shifts in 8 representative serum samples from patients with AKU at baseline, 12 and 24 months. (A) 9.5-7.4 ppm; (B) 7.5-5.5 ppm; (C) 5.5-4.9 ppm; (D) 4.5-2.5 ppm and (E) 2.5-0.5 ppm. In total there were 119 metabolite boundaries across the entire spectrum. Note there are no metabolite boundaries between 4.9-4.5 ppm as this region was removed due to water suppression.

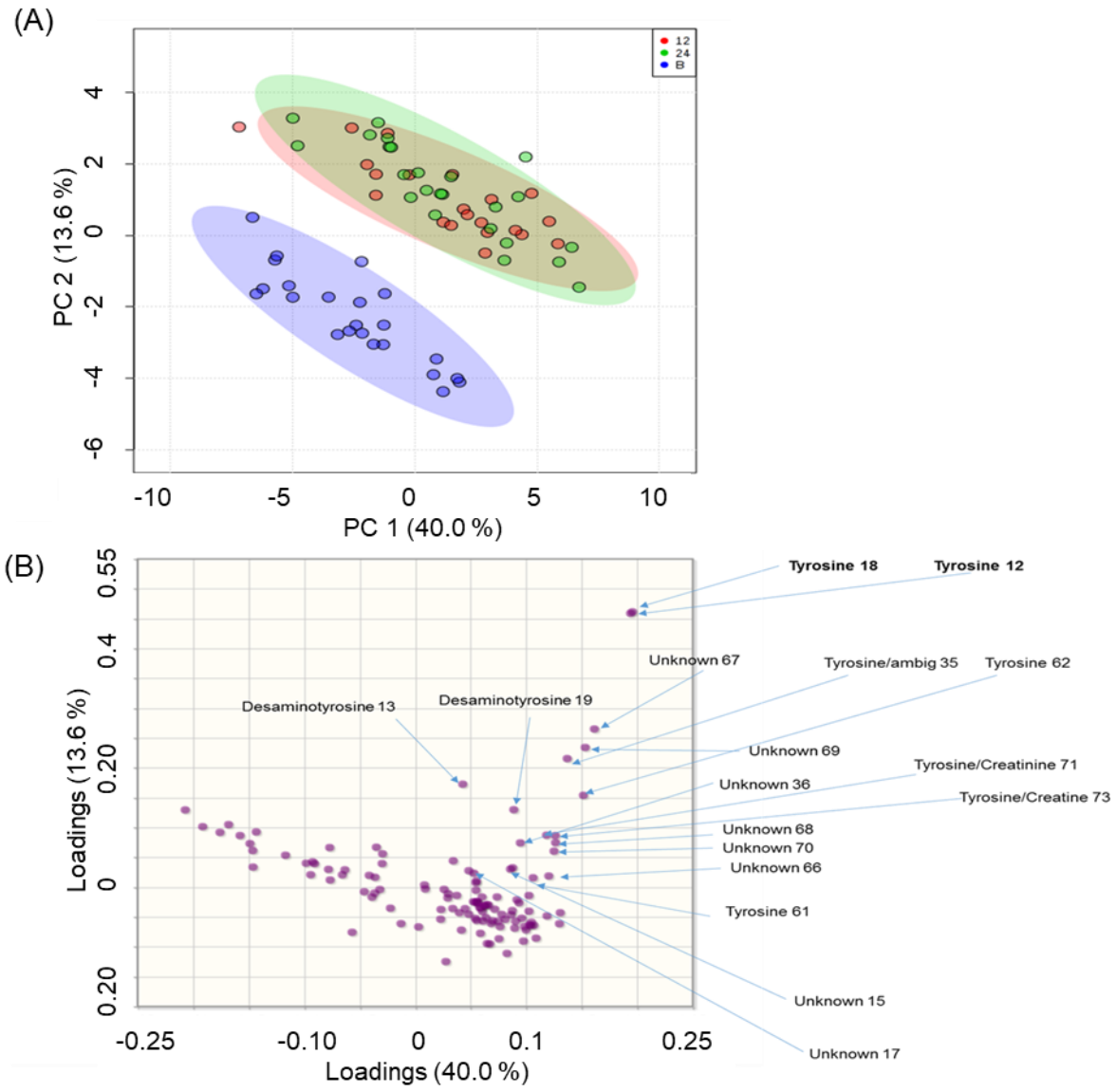

Figure 3A.5. Summary of statistical analysis of serum samples at baseline (pre-nitisinone, n=23), 12 months (n=21) and 24 months (n=24) following nitisinone treatment. (A) PCA and (B) Loadings associated with PCA. All labelled metabolites were deemed significant when baseline samples were compared to samples collected 12 and 24 months following nitisinone treatment. Metabolites in bold text had a $\log_2$ FC >2.

The majority of metabolite signals from the NMR analysis causing this separation were from tyrosine, specifically tyrosine 18 (at 7.2 ppm) and tyrosine 12 (6.9 ppm) (Figure 3A.6). Both signals showed a $\log_2$ FC increase

>2 (p values <0.05, Table 3A.1). Several unknown metabolite signals and desaminotyrosine are annotated in Figure 3A.5, but showed a $\log_2$ FC <2. The identity of the unknown metabolites was not determined as they were not significantly different. Of note, HGA was not observed in any samples from baseline (pre-nitisinone), 12 or at 24 months.

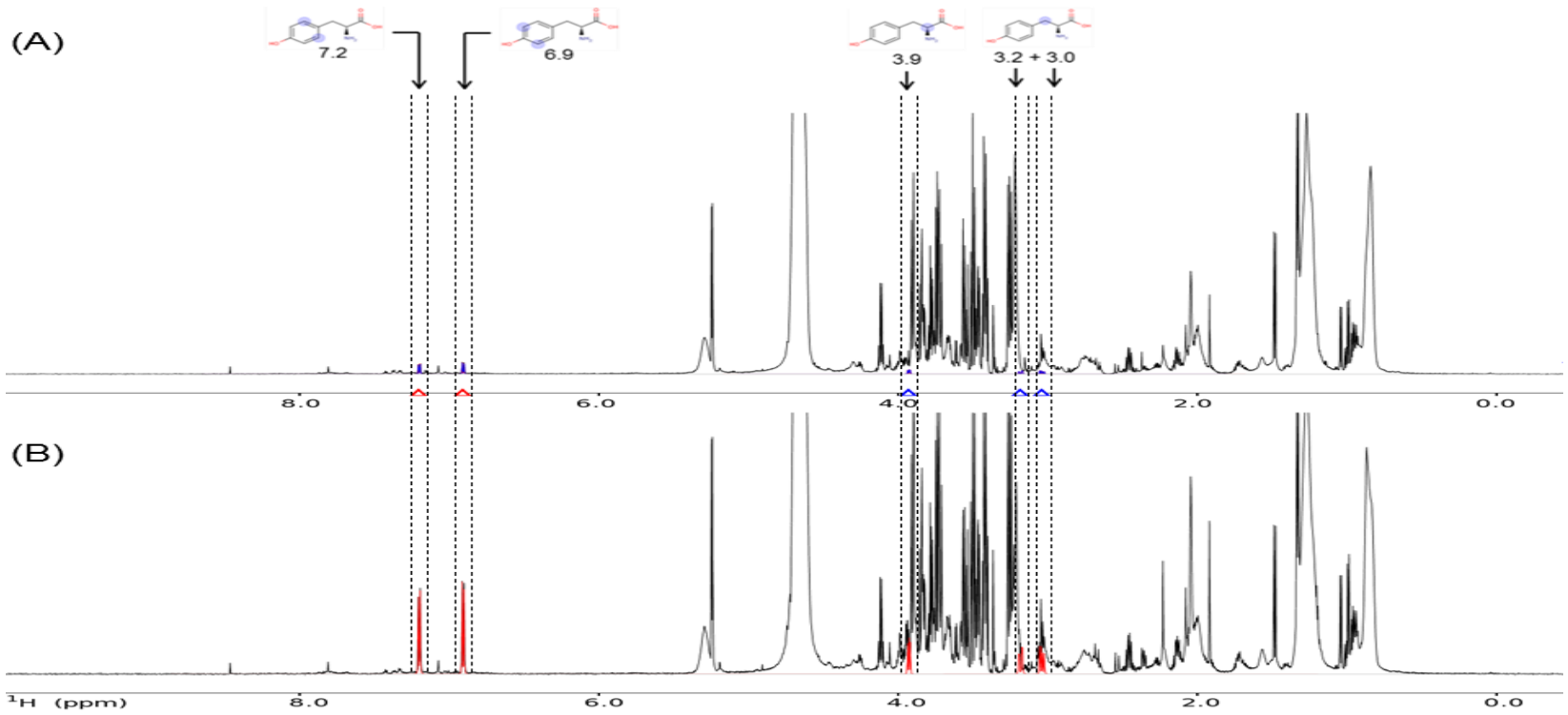

Figure 3A.6. ^{1}H NMR spectra from the analysis of serum taken from a patient (A) Pre-nitisinone and (B) 24 months after nitisinone treatment. In this figure the expected spectral shifts for tyrosine are highlighted (based on Chenomx library). The 5 shifts highlighted by purple rings on chemical structures srelate to the aromatic ring (7.2 and 6.9 ppm), amide group (3.9 ppm) and the alkyl groups (3.2 and 3.0 ppm). This figure shows the specific region of 0-8 ppm; x-axis relates to the spectral shift in ppm and the y-axis the intensity of the signal.

Metabolite	12 months		24 months	
	p value	Log_2 FC	p value	Log_2 FC
Tyrosine (tyrosine 18)	$7.82E^{-26}$	2.7	$1.03E^{-28}$	2.8
Tyrosine (tyrosine 12)	$1.03E^{-25}$	2.7	$1.29E^{-28}$	2.7

Table 3A.1. Serum metabolite changes identified post-nitisinone therapy at 12 and 24 months in patients with AKU using an in-house pattern file. Abundance expressed as log_2 FC compared to baseline (pre-nitisinone treatment). Log_2 FC included if >2 at 12 and or 24 months; p value <0.05 deemed significant.

3A.4 Discussion

The results presented herein were generated from 1D ^{1}H NMR experiments, which were performed in conjunction with LC-QTOF-MS analysis (Davison *et al.,* 2019b). The NMR data were not included in the peer reviewed publication presented in the main body of Chapter 3 and are included as an appendix for completeness.

This is the first study to evaluate the serum metabolome of patients with AKU following treatment with nitisinone, using NMR. The only other report of using NMR in the context of AKU relates to the diagnosis of AKU being made by NMR urinalysis (Yamaguchi *et al.,* 1986). As alluded to earlier the reason for using NMR in conjunction with LC-QTOF-MS was to maximise the coverage of the metabolome with a view to providing a more complete biological picture, and a structural identity in unknown entities that appear to change significantly following nitisinone therapy.

In total 119 metabolite spectral signals were identified in the serum samples analysed in this study, which corresponded to 37 matched metabolites in Chenomx (15 metabolites had multiple spectral shifts) and 27 unknown spectral shifts relating to a maximum of 27 metabolites, if it is assumed that

each metabolite had a single spectral shift. This is typical of NMR based metabolomic experiments in native serum where typically ~30 metabolites can be expected to be observed (Psychogios *et al.,* 2011). This is significantly lower than the number of metabolites matched using LC-QTOF-MS (see Chapter 3); 151 and 249 metabolites matched in positive and negative polarity, respectively. One of the reasons for this is that NMR is limited to detecting the most abundant metabolites (typically ≥1 micomolar). In contrast, mass spectrometry is able to measure femtomolar to attomolar concentrations, has significantly better resolution due to chromatographic separation and a greater dynamic range (~10^3–10^4). However, quantification is limited by ion suppression and when used in combination with liquid chromatography there are additional sources of complexity and error with mobile and stationary phases (Marshall *et al.,* 2017).

Another potential problem with NMR lies in the approach used to fit metabolite signals to patterns as many ^{1}H signals overlap (Markley *et al.,* 2017). In this study the impact of this was limited as standardised conditions and data collection protocols were used. Probabilistic fitting of data can further reduce the impact of this (Ravanbakhsh *et al.,* 2015).

The data presented herein confirms the significant hypertyrosinaemia that is known to occur following nitisinone therapy (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Davison *et al.,* 2018b; Davison *et al.,* 2018b), and is the first report of this using NMR. No HGA signals were observed in the data set, which is surprising as ^{1}H NMR reference spectra (600 MHz, 50 mmol/L in water, pH 7.0) on the Human Metabolome Database (http://www.hmdb.ca) show clear shifts at 3.47, 6.71, 6.81 ppm. There are several potential reasons for this, including sample preservation; HGA is known to be labile and sample acidification with perchloric acid is recommended (Hughes *et al.,* 2015). However, samples used in this set of experiments were not acidified due to the detrimental impact it would have on sample pH (pH 7.4 required for NMR analysis). Whilst HGA in the sample may have degraded to some extent, it is unlikely it would not be observed at

all as it is present in serum at 20-30 *μ*mol/L pre-nitisinone therapy (Phornphutkul *et al.,* 2002; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017).

Secondly the concentration of HGA may also have been grossly underestimated in serum due to it binding to serum proteins (Bell *et al.,* 1988; Nicholson *et al.,* 1989; Chatham *et al.,* 1999; Nagana Gowda *et al.,* 2014). High protein concentrations can also result in reduced transverse relaxation (T2) times for metabolite signals and facilitate exchange between protein bound and free metabolites, which result in broadened NMR peaks and poor quantitative accuracy (Nagana Gowda *et al.,* 2015).

Despite NMR not providing additional information on changes in identified metabolite abundance in this study it is a technique that is firmly entrenched in metabolomic studies. The advantages include minimal sample handling without the need for chromatography, that it is easily quantitative, and provides multiple means of metabolite identification.

3A.5 Conclusions

This is the first study to evaluate the serum metabolome of patients with AKU following treatment with nitisinone using NMR, and confirms the presence of hypertyrosinaemia following treatment with nitisinone. This study highlights the challenges associated with the analysis of serum samples that have not undergone clean up prior to NMR analysis. Further identification of unknown serum metabolites present in the clinical samples is required to exploit all additional information provided in this dataset.

3A.6 Supplementary material

Shift (ppm)		Metabolite name	Metabolite number
Left	Right		
8.47	8.46	Formate	1
8.213	8.186	Unknown	2
7.898	7.851	Unknown	3
7.85	7.83	Unknown	4
7.82	7.8	Histidine	5
7.739	7.644	Unknown	6
7.448	7.409	Phenylalanine	7
7.3893	7.374	Overlap1/phenylalanine	8
7.3739	7.363	Overlap2/phenylalanine	9
7.3629	7.347	Overlap3/phenylalanine	10
7.3469	7.3191	Phenylalanine	11
7.22	7.178	Tyrosine	12
7.166	7.1298	Desaminotyrosine	13
7.0842	7.055	Histidine	14
7.05	7.0195	Unknown	15
7.0194	6.996	Unknown	16
6.9959	6.923	Unknown	17
6.9223	6.8802	Tyrosine	18
6.8801	6.75	Desaminotyrosine/n-acetyltyrosine	19
5.362	5.253	Mobile-lipid	20
5.253	5.226	Glucose	21
5.219	5.1671	Fucose	22
4.9161	4.9055	4-Hydroxy-3-methoxymandelate	23
4.3317	4.2225	Unknown	24
4.1888	4.1461	Fucose	25
4.1436	4.1363	Glutarate	26
4.1361	4.1262	Lactate/glutarate	27
4.1261	4.0922	Lactate	28
4.0918	4.064	Fucose	29
4.065	4.0514	Creatinine	30
4.02	3.9991	Amino acid-α-backbone	31
3.9992	3.9807	Amino acid-α-backbone	32
3.9803	3.9701	Unknown	33
3.9699	3.9544	Galactitol	34
3.9551	3.9291	Tyrosine	35
3.929	3.9258	Unknown	36
3.9206	3.9084	Glucose	37
3.9083	3.8876	Glucose	38
3.8878	3.8704	Unknown	39
3.8677	3.8524	Glucose	40
3.8524	3.82	Glucose	41
3.8149	3.8059	Unknown	42

3.8046	3.7954	Fucose	43
3.7953	3.7558	Alanine/glucose	44
3.7559	3.7199	Glucose	45
3.7199	3.7	Unknown	46
3.699	3.6292	Amino acid-α-backbone	47
3.6275	3.6032	Valine	48
3.5971	3.5767	Amino acid-α-backbone	49
3.5763	3.5642	Glycine	50
3.5637	3.5284	Glucose	51
3.529	3.4555	Glucose	52
3.4553	3.4465	Unknown	53
3.4456	3.3859	Glucose	54
3.3858	3.3783	3,4-Dihydroxybenzeneacetate	55
3.376	3.365	Unknown	56
3.3648	3.3259	Unknown	57
3.3083	3.2786	N-carbamoyl-alanine	58
3.2782	3.2393	Glucose	59
3.2378	3.209	Tyrosine	60
3.208	3.19	Tyrosine	61
3.189	3.1722	Tyrosine	62
3.1721	3.1548	N-nitrosodimethylamine	63
3.1547	3.1332	Malonate	64
3.1246	3.1052	Cis-aconitate	65
3.0833	3.0746	Unknown	66
3.0745	3.0684	Unknown	67
3.0684	3.0634	Unknown	68
3.0633	3.057	Unknown	69
3.057	3.0536	Unknown	70
3.0535	3.047	Tyrosine/creatinine	71
3.0469	3.0427	Tyrosine/creatine-phosphate	72
3.0426	3.032	Tyrosine/creatine	73
3.031	3.0	Tyrosine	74
2.9959	2.9485	Asparagine	75
2.9484	2.9283	Asparagine	76
2.9252	2.8846	Unknown	77
2.8737	2.8603	Asparagine	78
2.86	2.8477	Asparagine	79
2.8475	2.8298	Methyl-guanidine	80
2.8297	2.8123	Unknown/acetyltyrosine	81
2.812	2.77	Mobile-lipid	82
2.769	2.7357	Mobile-lipid	83
2.7355	2.7104	Sarcosine	84
2.7104	2.69	Unknown	85
2.6899	2.68	Unknown	86
2.679	2.658	Citrate	87
2.6565	2.6281	Unknown/n-carbomoylaspartate	88
2.5776	2.5437	Citrate	89
2.5435	2.5137	Citrate	90

2.5125	2.4859	Glutamine	91
2.4857	2.4399	Glutamine/n-carbamoylaspartate	92
2.3893	2.3809	3-Hydroxyisovalerate	93
2.3809	2.34	Glutamate	94
2.339	2.3109	Unknown	95
2.3108	2.2566	Valine	96
2.2564	2.2401	Mobile-lipid	97
2.24	2.1884	Mobile-lipid	98
2.1883	2.1	Glutamine/glutamic acid	99
2.0992	2.0708	Mobile-lipid/glycoprotein	100
2.07	2.0297	Mobile-lipid/proline	101
2.0295	1.9404	Unknown	102
1.9363	1.9024	Acetate	103
1.7727	1.63	Leucine	104
1.62	1.53	Mobile-lipid	105
1.507	1.46	Alanine	106
1.44	1.389	Biotin	107
1.38	1.32	Lactate	108
1.3198	1.2182	Mobile-lipid	109
1.2181	1.206	Unknown	110
1.205	1.18	Fucose	111
1.1799	1.0652	Unknown	112
1.0651	1.031	Valine	113
1.026	1.0065	Isoleucine	114
1.0063	0.985	Valine	115
0.985	0.95	Leucine	116
0.9498	0.92	Isoleucine	117
0.919	0.8605	Mobile-lipid	118
0.8604	0.8	Mobile-lipid	119

Appendix Supplementary Table 3.1. Pattern file generated in Amix for serum samples obtained from AKU patients pre and post treatment with nitisinone. Metabolite names were assigned based on matches from Chenomx (Version 8.2, Canada), which contains 328 metabolites. Metabolites that were not matched were denoted as unknown.

3A.7 Declaration and acknowledgements

Preparation, analysis and data processing associated with the serum samples from patients attending the NAC was performed by Andrew Davison and Brendan Norman (Department of Musculoskeletal Biology I, University of Liverpool, UK).

Jean Devine (Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Royal Liverpool Hospital, Liverpool, UK) processed all serum samples from patients attending the NAC.

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THE UNIVERSITY
of LIVERPOOL

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

Biochemical investigations into the impact of nitisinone treatment on the metabolome and assessment of whether nitisinone induced hypertyrosinaemia alters neurotransmitter metabolism

Volume II

Andrew Stuart Davison

January 2020

Part III

Assessment of the impact of nitisinone on: (1) serum amino acids and urinary monoamine neurotransmitter metabolites, and BDI-II scores in patients with alkaptonuria, and (2) neurotransmitter patterns in the brain and cerebrospinal fluid of a murine model of alkaptonuria

Chapter 4

Serum amino acid profiling in patients with alkaptonuria before and after treatment with nitisinone

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4.1 Abstract

Background: Alkaptonuria (AKU) is a rare inherited disorder of tyrosine metabolic pathway. Our group is evaluating the use of the homogentisic acid lowering agent nitisinone in patients with AKU. A major biochemical consequence of this treatment is hypertyrosinaemia. Herein we report the concentration of 20 serum amino acids over a 36-month period pre- and post-treatment with nitisinone.

Materials and methods: Fasting serum samples were collected at baseline (pre-nitisinone), 3 (2 mg nitisinone every other day), 6, 12, 24 and 36 (2 mg nitisinone daily) months. Amino acids were measured using the Biochrom-30 high performance liquid chromatography cation exchange system with ninhydrin detection.

Results: Fifty patients [21 female, mean age (±standard deviation) 54.1(15.6) years (range 25-75); 29 male, mean age 49.3(11.6) years (range 22-70 years)] were included. Following treatment mean tyrosine concentrations increased 7-8 fold (baseline 69.8 μmol/L; 3 months 670.7 μmol/L; 6 months: 666.4 μmol/L; 12 months 692.9 μmol/L; 24 months 649.4 μmol/L; 36 months 724.8 μmol/L, $p<0.001$ for all visits compared to baseline). At baseline mean phenylalanine, aspartic acid and arginine concentrations were outside the normal reference range. Following treatment the ratios of phenylalanine:tyrosine, phenylalanine:large neutral amino acids, arginine:branched chain amino acids and branched chain:aromatic amino acids decreased ($p<0.05$), and the tyrosine:large neutral amino acid ratio increased ($p<0.0001$).

Conclusions: Marked hypertyrosinaemia was observed following treatment with nitisinone. Noteworthy changes were also observed in the ratio of several amino acids following treatment with nitisinone suggesting that the availability of amino acids for neurotransmitter biosynthesis, and liver function may be altered following treatment with nitisinone.

4.2 Introduction

Alkaptonuria (AKU, OMIM 203500) is a rare autosomal recessive disorder of the tyrosine metabolic pathway, resulting from a congenital deficiency in the enzyme homogentisate-1,2-dioxygenase (HGD, E.C.1.12.11.5). AKU occurs 1 in 250 000 of the general population (Phornphutkul *et al.,* 2002), but in certain countries it is observed more commonly; for instance in Slovakia it is estimated to occur in 1 in 19 000 of the population (Milch, 1960; Zatkova, 2011).

One of the major metabolic implications of AKU is that the circulating concentration of homogentisic acid (HGA) significantly increases. This pathognomonic finding is thought to be causally responsible for a number of abnormalities observed in the disease. These include spondyloarthropathy, characterised by progressive kyphoscoliosis and impaired spinal and thoracic mobility, as well as renal and prostate stones, aortic valve stenosis, osteoporosis, fractures, and ruptures of tendons, ligaments and muscle (Ranganath *et al.,* 2013).

Supportive medical management of AKU is the mainstay of treatment and includes a low protein diet, analgesia and arthroplasty (Ranganath *et al.,* 2013). A newer unlicenced treatment for AKU is the drug nitisinone, a competitive reversible inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C. 1.13.11.27). It reduces the formation of HGA and thus has the potential to prevent or slow the complications observed in patients with AKU. Previous studies (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017) have demonstrated the clear biochemical impact of nitisinone treatment, all demonstrating a >94 % reduction in urinary HGA. However, none have shown improvement in clinical parameters. This may be for several reasons, including: (1) AKU is a slow progressive disorder and thus study duration may not have been not long enough to demonstrate improvement, (2) the optimal dose of nitisinone may not used and (3) clinical outcomes measured may not have been appropriate.

At the National Alkaptonuria Centre (NAC) in Liverpool nitisinone is being used off licence (patients are given 2 mg of nitisinone daily, off-licence) for treatment of AKU for all patients in the UK, as it is recognised that nitisinone is a potential treatment for this debilitating disease. At the NAC it has also been demonstrated in a real life setting that this treatment can reduce urinary HGA by 94 % (Milan *et al.,* 2017).

In contrast, nitisinone is already licenced for the treatment of hereditary tyrosinaemia type 1 (HT1, OMIM 276700) and has proved to be a very efficacious mode of treatment (McKiernan, 2013; McKiernan *et al.,* 2015).

One of the major metabolic consequences of treating patients with nitisinone in AKU and HT1 is that circulating tyrosine concentrations markedly increase (Lindstedt *et al.,* 1992; Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; McKiernan *et al.,* 2015; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; van Ginkel *et al*., 2016; van Ginkel *et al*., 2017; Milan *et al.,* 2017).

Hypertyrosinaemia has been observed in patients with AKU that attend the NAC in Liverpool and during the Suitability Of Nitisinone In Alkaptonuria 1 (SONIA-1) clinical trial (Ranganath *et al.,* 2016), which evaluated the efficacy of different daily doses of nitisinone over a 4 week period. Beyond this there is nothing reported on the wider impact of nitisinone on amino acid metabolism.

For many years there has been keen interest in amino acid metabolism in health and disease, beyond the traditional setting of inborn errors of metabolism. This is because amino acids are not only basic metabolites, but are also key regulators in many metabolic pathways. Examples of areas studied include cognitive function and Alzheimer's disease (Ravaglia *et al.,* 2004), gastroesophogeal cancer (Crotti *et al.,* 2017), diabetes (Bi *et al.,* 2017) and aortic dissection (Wang *et al.,* 2017).

In addition, while there have been several studies evaluating serum and plasma amino acid concentrations both in men and women of a variety of ages (Armstrong *et al.,* 1973a; Armstrong *et al.,* 1973b; Armstrong *et al.,* 1973c; Rudman *et al.,* 1989; Caballero *et al.,*1991; Sarwar *et al.,* 1991; Chan, 1999; Pitkänen *et al.,* 2003; Kouchiwa *et al.,* 2012; Davison *et al.,* 2015), none have reported fasting serum amino acid concentrations in patients with AKU pre- and post-nitisinone therapy.

For the first time, this longitudinal survey reports the concentration of essential amino acids (valine, leucine, isoleucine, threonine, methionine, tryptophan, phenylalanine, lysine and histidine) and non-essential amino acids (aspartic acid, asparagine, glutamic acid, glutamine, alanine, glycine, cystine, arginine, tyrosine, serine, and proline) in patients that have attended the NAC with AKU pre- and post-nitisinone therapy over a 36 month period.

4.3 Materials and methods

4.3.1 Patients

4.3.1.1 Protocol for patients that attend the NAC for treatment with nitisinone

The protocol for treatment at the NAC is that patients with confirmed AKU are commenced on a 2 mg dose of nitisinone, on alternative days for the first 3 months, which is then increased to 2 mg daily thereafter. Assessments are repeated on an annual basis to monitor response to therapy. Nitisinone is given off licence to investigate its safety and efficacy. It is hypothesised that if HGA levels are reduced before the onset of overt ochronosis, this might prevent or stop the development of the debilitating features observed in AKU.

Inclusion criteria are that individuals must have the diagnosis of AKU; are residents of England or Scotland, and over the age of 16 years. Confirmed diagnosis of AKU is based upon increased urinary excretion of HGA (urine HGA excretion in healthy volunteers has been demonstrated to be <2.92

*μ*mol/day (Davison *et al.,* 2015)) and is mandatory for referral to the NAC. Exclusion criteria are individuals must not be pregnant and or lactating. All patients are provided with written information about the scope of the Centre and the assessments they will receive. All patients at the NAC have biochemical measurements with clinical assessments performed at baseline, day 4 (2 days post-nitisinone), 3 months, 6 months and 12 months, with annual monitoring thereafter.

4.3.1.2 Ethical Approval

Data collection and analyses at the NAC has approval from the Royal Liverpool and Broadgreen University Hospital Trust Audit Committee (Audit no. ACO3836). This is not a clinical trial and therefore ethical approval was not required. Data obtained is following standard clinical assessments during the course of providing a service upon referral to the NAC. Patients are informed verbally and through patient information leaflets about the activities of the NAC. Patients are also explicitly informed that data may be used for publication and within the NAC patient information leaflet the following paragraph is included:

"We could publish results from the study but if we do, we will make sure that you cannot be identified in anyway. All data used for publicity or for other research purposes will ensure total anonymity. Please let us know when you are visiting the NAC that you understand and have no objections to this." No patient has objected to the use of their data.

4.3.1.3 Subjects included in the study

To date (November 2017), 62 patients with AKU have been enrolled at the NAC for treatment with nitisinone.

Twelve of the 62 patients were excluded from this survey; 7 were Welsh patients and therefore funding was not available for treatment with nitisinone; 3 patients were on nitisinone prior to enrolment at the NAC and 2 patients were not receiving a standard nitisinone treatment regimen due to corneal keratopathy (Khedr *et al.,* 2018).

Fifty patients [21 female, mean age (±standard deviation) 54.1(15.6) years (range 25-75); 29 male, mean age 49.3(11.6) years (range 22-70 years)] were included in this 36 month longitudinal survey reporting the biochemical data obtained from monitoring 20 serum amino acids. This is an on-going service and at the time of preparing this manuscript results were not available for all 50 patients at each time point, apart from baseline. Serum amino acid results are included from baseline (pre-nitisinone), 3 (2 mg nitisinone every other day), 6 (2 mg nitisinone daily), 12 (2 mg nitisinone daily), 24 (2 mg nitisinone daily) and 36 (2 mg nitisinone daily) months. Results are not included after baseline in all patients as individuals have attended the NAC for different durations and not all patients have attended planned visits to the NAC.

4.3.2 Analytical Methods

4.3.2.1 Sample collection

Serum samples were collected from patients (S-monovette, Sarstedt, Germany), centrifuged (10 min at 3000 rpm) and stored at -20 °C until analysis. All serum samples were collected following an overnight fast (at least 8 h). Patients' dietary intake of protein was not restricted during this study.

4.3.2.2 Measurement of serum amino acids

Serum amino acid concentrations were determined using the Biochrom 30 high performance liquid chromatography cation exchange system with ninhydrin detection (Biochrom, Cambridge, UK). Amino acids were applied to the high pressure PEEK column packed with Ultrapac 8 cation exchange resin (Biochrom, Cambridge). The conditions of the analytical column were altered using a series of lithium buffers [lithium Buffer 1 (Biochrom, product code: 80-2038-15); lithium buffer 2 (Biochrom, product code: 80-2038-16); lithium Buffer 3 (Biochrom, product code: 80-2099-83); lithium Buffer 4 (Biochrom 80-2097-18); lithium Buffer 5 (Biochrom, product code 80-2037-69); lithium regeneration buffer 6 (Biochrom, product code: 80-2038-20)]. Altering buffer composition over the 150 min analytical run increases pH until

the iso-ionic point of the different amino acids are reached, facilitating elution of amino acids from the analytical column.

Detection was achieved using a post column reaction system, the eluent being mixed with an ultra ninhydrin solution in a ratio of 2:1 (Biochrom, product code: 80-2117-64) and heated to 135 °C. The ninhydrin amino acid complex was monitored at 2 wavelengths to detect amino and imino acids. Amino acids underwent a reaction with ninhydrin (triketohydrindene) to form a specific blue/purple colour complex with an absorbance maximum at 570 nm (monitored in channel A). The intensity of the reaction product was proportional to the amino acid concentration. Imino acids do not have free amino groups so do not react in the same way, but form a yellow coloured complex with an absorbance maximum at 440 nm. Their absorbance was monitored on channel B.

4.3.2.3 Calibration and internal standards

Calibration was performed using a series of aqueous standards purchased from Sigma (Dorset, UK) [amino acids basic standard (product code: A6282); amino acids acidic/neutral standard (product code: A6407); glutamine (product code: G8540)]. Stock calibrations standards were made to 2.0 mmol/L, except glutamine which was made to 2.5 mmol/L. All working calibrator concentrations were then diluted with loading buffer (Biochrom, product code: 80-2038-70) to a final concentration of 100 μmol/L, except for cystine which was 50 μmol/L. The diluted working calibrator also contained the internal standards S-2-amino-ethyl-L-cystine hydrochloride (product code: A2636) and norleucine (product code: N6877) at a final concentration of 200 μmol/L and 5-sulphosalicylic acid at a final concentration of 91.7 mmol/L.

4.2.3.4 Internal quality control

ClinCheck® plasma control (level 1) for amino acids was used as internal quality control material (Recipe, Germany). This contained all amino acids measured, apart from cystine, which was purchased from Sigma (Dorset, UK) to make in-house internal quality control material. Inter-assay coefficient of variation for all 20 amino acids was <4 %; this is detailed below as mean

amino acid concentration (*μ*mol/L) (±standard deviation), coefficient of variation (%). Aspartic acid: 12 (0.5), 3.9; threonine: 109.3 (1.9), 1.8; serine: 96.7 (1.5), 1.5; asparagine: 45 (1.1), 2.3; glutamic acid: 98 (1.2), 1.3; glutamine: 408.9 (9.1), 2.2; glycine: 185.3 (3.0), 1.6; alanine: 405.9 (8.6), 2.1; valine: 233.2 (5.1), 2.2; cystine: 13.2(0.3), 2.4; methionine: 27.9 (0.9), 3.1; isoleucine: 76.8 (2.3), 3.0; leucine: 139.4 (3.1), 2.2; tyrosine: 68.3 (1.7), 2.5; phenylalanine: 78.1 (1.8), 2.3; lysine: 171.8 (3.0), 1.7; histidine: 73.5 (1.7), 2.3; tryptophan: 53.5 (1.0), 1.8; arginine: 30.4 (0.8), 2.8; proline: 236.2 (3.7); 3.7.

4.2.3.5 Sample preparation

100 *μ*L of calibrator/serum/internal quality control material was mixed with 100 *μ*L of a solution containing sulfosalicylic acid (183.4 mmol/L) to precipitate proteins and internal standards (S-2-amino-ethyl-L-cystine hydrochloride and norleucine both at 100 *μ*mol/L). Samples were then put in the fridge at 4 °C for 1 h, and then centrifuged at 13 000 rpm for 10 min. 60 *μ*L of supernatant was injected onto the analytical column for analysis.

4.2.3.6 Data Analysis and calculations

Amino acid concentrations were calculated off-line using EZCHrom Elite software (Build 3.2.1 Agilent, USA). All statistical analysis were performed using Graphpad Instat (Version 3.10, 2009, CA, USA) and Analyse It for Microsoft Excel (Version 2.20 Analyse-it Software, Ltd, Leeds, UK).

Kolmogorov-Smirnov testing was performed to assess if serum amino concentrations were normally distributed. For non-parametric data a Kruskal-Wallis test with a Dunn's multiple comparisons post-hoc test was used to assess significant differences in serum amino acid concentrations pre- and post-treatment with nitisinone. For parametric data a 1-way ANOVA with Tukey post-hoc was applied. A p value <0.05 was deemed significant.

4.2.3.7 Amino acid ratios

All amino acid concentrations used in calculations were in *μ*mol/L. Ratios were calculated to assess dietary adequacy of protein intake (calculation a) (Antener *et al.,* 1981); to predict phenylalanine, tyrosine, and arginine

availability to the brain for neurotransmitter synthesis (calculations b-d) (Lieberman, 1999; Ravaglia *et al*., 2004) and to assess liver metabolism, hepatic functional reserve and the severity of liver damage (calculation e) (Fisher *et al.,* 1976).

(a) Phenylalanine to tyrosine = [phenylalanine]/[tyrosine]

(b) Tyrosine to large neutral amino acids ([tyrosine]:[LNAA]) = [tyrosine]/([tyrosine] + [phenylalanine] + [tryptophan] + [leucine] + [isoleucine] +[valine])

(c) Phenylalanine to large neutral amino acids ([phenylalanine]:[LNAA]) = [phenylalanine]/([tyrosine] + [phenylalanine] + [tryptophan] + [leucine] + [isoleucine] +[valine])

(d) Arginine to branched chain amino acids ([arginine]:[BCAA]) = [arginine]/([leucine] + [isoleucine] +[valine])

(e) Branched chain amino acids to aromatic amino acids ([BCAA]:[AAA]) = ([leucine] + [valine] + [isoleucine])/ [phenylalanine] + [tyrosine])

4.3 Results

4.3.1 Serum amino acid concentrations pre-nitisinone treatment

Table 4.1 summarises mean (±standard deviation) fasting serum amino acid concentrations from all patients included in this longitudinal survey. Fifty patients had amino acids measured at baseline (Table 4.1). After baseline amino acids were not measured in all patients as individuals have attended the NAC for different durations and not all patients attended planned visits to the NAC.

Mean amino acid concentrations were within the normal reference range at baseline (pre-nitisinone treatment) apart from phenylalanine, aspartic acid,

arginine and cystine (Table 4.1). The latter being the only amino acid below the lower reference range, despite there being a large spread of concentrations. All other amino acids detailed were outside the upper reference range. Following treatment with nitisinone these amino acids did not change significantly over the 36 month period studied.

4.3.2 Impact of nitisinone treatment on serum amino acid concentrations

Mean serum tyrosine and tryptophan concentrations were within the normal reference range pre-nitisinone treatment, and phenylalanine was marginally outside the normal reference range (Table 4.1). Following treatment with 2 mg nitisinone, every other day (3 month visit) and daily (all visits after 3 month), tyrosine concentrations increased to 7-8 fold ($p<0.001$, at all visits).

Phenylalanine and tryptophan concentrations were not significantly different over the 36 month period studied when compared to baseline. Phenylalanine concentrations did however show an increasing concentration.

Several amino acid ratios were calculated (calculations a-e) (Table 4.1). All amino acid ratios were within the normal range pre-nitisinone treatment, except the phenylalanine to tyrosine ratio, which was increased. Following treatment with nitisinone all amino acid ratios showed a significant decrease (see Table 4.2 for p values, all were <0.05), apart from the tyrosine to LNAA ratio which showed a significant increase ($p<0.0001$).

Amino acid (Reference range, μmol/L)	Baseline (n=50)	3 months (n=37)	6 months (n=29)	12 months (n=37)	24 months (n=34)	36 months (n=20)
Phenylalanine (30-76)[E,1]	87.42 (13.9)	84.4 (18.3)	91.5 (22.5)	94.5 (13.5)	92.9 (13.6)	97.6 (15.0)
Tyrosine (29-92)[1]	69.8 (15.7)	670.7 (155.0)	666.4 (162.3)	692.9 (119.8)	649.4 (183.6)	724.8 (116.8)
Tryptophan (40-79)[E,1]	51.8 (11.5)	50.9 (9.9)	48.5 (11.9)	48.1 (11.1)	51.1 (10.2)	53.5 (14.7)
Glutamine (326-800)[2]	571.2 (108.0)	476.2 (79.4)	495.1 (81.1)	527.6 (86.0)	525.4 (77.8)	469.4 (103.5)
Asparagine (30-70)[2]	72.1 (15.1)	64.7 (14.6)	65.3 (17.9)	69.2 (11.0)	61.3 (11.8)	61.4 (9.1)
Aspartic acid (2-5)[3]	39.8 (9.7)	38.2 (10.9)	37.9 (9.9)	38.1 (6.7)	39.5 (8.7)	45.4 (17.0)
Glutamic acid (26-151)[3]	128.9 (40.4)	129.2 (56.6)	141.1 (63.5)	151.8 (37.4)	137.5 (32.4)	149.5 (49.2)
Cystine (36-61)[4]	11.1 (9.3)	6.9 (4.6)	8.3 (8.4)	9.6 (9.1)	13.9 (9.1)	15.7 (13.5)
Methionine (10-41)[E,4]	25.5 (6.6)	21.8 (4.5)	23.7 (5.9)	25.8 (5.4)	24.4 (4.3)	25.2 (5.3)
Glycine (120-436)[5]	316.7 (77.6)	299.5 (60.6)	330.5 (61.6)	343.2 (63.2)	329.9 (75.1)	332.6 (74.4)
Isoleucine (20-91)[E,5]	69.7 (17.6)	63.2 (15.5)	64.5 (15.7)	64.8 (12.1)	66.6 (13.5)	72.5 (18.0)
Leucine (44-169)[E,5]	148.8 (27.8)	132.1 (28.4)	137.1 (30.7)	139.4 (21.6)	139.7 (20.7)	134.6 (33.1)
Proline (66-330)[5]	204.3 (89.6)	183.3 (76.3)	207.9 (108.9)	180.4 (63.5)	182.3 (53.3)	182.5 (75.8)
Valine (79-313)[E,5]	240.4 (43.4)	222.9 (44.6)	226.7 (45.4)	234.2 (36.0)	241.9 (40.2)	250.3 (52.7)
Alanine (112-529)[5]	462.0 (96.9)	444.8 (92.0)	485.1 (124.8)	427.8 (83.3)	448.5 (82.6)	450.6 (79.5)
Histidine (43-111)[E,6]	84.1 (12.3)	85.0 (12.8)	86.7 (16.5)	80.7 (9.5)	85.2 (11.7)	79.3 (10.0)
Lysine (66-242)[E,6]	191.3 (32.2)	180.5 (33.8)	180.45 (38.8)	177.8 (27.9)	183.8 (28.0)	185.6 (27.4)
Arginine (14-102)[6]	105.2 (23.4)	113.9 (31.1)	116.2 (26.9)	98.1 (13.8)	96.6 (15.9)	108.2 (18.0)
Serine (69-206)[7]	162.3 (28.7)	149.8 (21.9)	155.5 (29.6)	161.3 (18.6)	157.9 (30.5)	162.4 (25.6)
Threonine (43-218)[E,7]	132.2 (27.5)	114.5 (25.5)	112.7 (27.0)	112.5 (21.3)	113.4 (23.6)	124.5 (27.8)
Phenylalanine:tyrosine (0.80-1.0)	1.28 (0.21)	0.13 (0.04)	0.13 (0.03)	0.14 (0.03)	0.16 (0.1)	0.13 (0.04)

Tyrosine:LNAA (0.0-0.14)	0.10 (0.01)	0.55 (0.07)	0.54 (0.10)	0.54 (0.05)	0.52 (0.09)	0.54 (0.06)
Phenyalanine:LNAA (0.09-0.13)	0.13 (0.01)	0.07 (0.01)	0.07 (0.02)	0.07 (0.01)	0.08 (0.02)	0.07 (0.01)
Arginine:BCAA (0.16-0.23)	0.23 (0.05)	0.27 (0.07)	0.27 (0.07)	0.23 (0.04)	0.22 (0.05)	0.24 (0.04)
BCAA/AAA (Fishers ratio) (2.7-3.5)	2.93 (0.37)	0.56 (0.14)	0.71 (0.86)	0.56 (0.09)	0.71 (0.49)	0.56 (0.13)

Table 4.1. Mean serum amino acid concentrations (mean(±standard deviation), *µ*mol/L) in patients attending the NAC over a 36 month period. All samples were collected in a fasted state (overnight fast >8 h). Baseline – refers to pre-nitisinone therapy; 3 months patients received 2 mg nitisinone every other day; after 3 months patients received 2 mg nitisinone daily. Aromatic[1]; amidic[2]; acidic[3]; sulphur containing[4]; aliphatic[5]; basic[6]; hydroxylic[7]; essential[E] amino acids. Amino acid reference ranges were determined in-house. Amino acid ratio reference ranges were adopted from Antener *et al.* (1981); Lieberman, 1999; Ravaglia *et al.* (2004); Kouchiwa *et al.* (2012). LNAA – large neutral amino acids; BCAA – branched chain amino acids; AAA – aromatic amino acid.

4.3.3 Amino acid concentrations showing differences after nitisinone treatment started

Table 4.2 summarises significant differences in amino acid concentrations that were observed between visits after nitisinone treatment was started. Apart from tyrosine, the amino acids asparagine and glutamine showed significant decreases in concentration post nitisinone treatment at 24 and 36 months (p=0.004), and 3, 6 and 36 months respectively (p<0.0001).

Amino acid	p value	Change in amino acid concentration observed compared to baseline	Change observed
Asparagine	0.004	24 and 36 months	Decrease
Glutamine	<0.0001	3, 6 and 36 months	Decrease
Tyrosine	<0.0001	3, 6, 12, 24 and 36 months	Increase
Phenylalanine:tyrosine	<0.0001	3, 6, 12, 24 and 36 months	Decrease
Tyrosine:LNAA	<0.0001	3, 6, 12, 24 and 36 months	Increase
Phenyalanine:LNAA	<0.0001	3, 6, 12, 24 and 36 months	Decrease
Arginine:BCAA	<0.01 <0.05	3 months 6 months	Decrease
BCAA/AAA (Fishers Ratio)	<0.0001	3, 6, 12, 24 and 36 months	Decrease

Table 4.2. Serum amino acid concentrations that were significantly different in patients attending the NAC over a 36 month period. Baseline – refers to pre-nitisinone therapy; 3 months patients received 2 mg nitisinone every other day; after 3 months patients received 2 mg nitisinone daily. LNAA – large neutral amino acids; BCAA – branched chain amino acids; AAA – aromatic amino acid. p value <0.05 deemed significant.

4.4 Discussion

Herein for the first time we report the concentration of 20 serum amino acids over 36 month period in a large cohort of patients with AKU before and after treatment with nitisinone. This highly unique longitudinal survey examines the impact of AKU and its treatment on amino acid metabolism in a real life

healthcare setting. Traditionally the focus has been on the tyrosine metabolic pathway when studying AKU, and several reports have shown that patients treated with nitisinone have marked hypertyrosinaemia (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017). In line with previous authors we too observed a significant increase in serum tyrosine following treatment with nitisinone. This occurs because nitisinone is a reversible competitive inhibitor of HPPD, and essentially creates a new metabolic defect up-stream of its site of action. The biochemical pattern observed is that which is seen in type 3 hereditary tyrosinaemia (OMIM 276710), so called 'pseudo-type 3 tyrosinaemia'.

Recently Milan *et al.* (2017) reported tyrosine concentrations in patients treated with nitisinone over a 24 month period. In contrast, this longitudinal survey reports the concentration of 20 serum amino acids, not just tyrosine over a 36 month period to establish the wider impact of treatment with nitisinone on amino acid metabolism. Moreover data presented herein is in a larger cohort of patients.

Concerns exist over the impact of hypertyrosinaemia, as tyrosine is transported across the blood brain barrier and may result in high brain tyrosine concentrations (Thimm *et al.,* 2011), and thus increased dopamine (DP) concentrations in cerebrospinal fluid (CSF) as tyrosine is its precursor. A previous study has demonstrated an increase in homovanillic acid (DP metabolite) concentrations in CSF (Thimm *et al.,* 2011), which suggests that DP metabolism may be altered. Moreover animal studies have also shown a direct neurotoxic effect of tyrosine, including oxidative stress (Macedo *et al.,* 2013) and altering DNA repair (Da Pra *et al.,* 2014).

Changes in the other aromatic amino acids were fairly unremarkable. Serum tryptophan remained within the normal reference range over the 36 month period. Previous studies in patients with HT1 treated with nitisinone, and in animal models have suggested that hypertyrosinaemia observed following treatment may reduce the biosynthesis of serotonin, and thus be responsible

for altered cognitive function and behavioral problems observed in HT1 (De Laet *et al.,* 2011; Thimm *et al.,* 2012; Masurel-Paulet *et al.,* 2013; Bendadi *et al.,* 2014; van Ginkel *et al.,* 2016). Evidence of this is sparse and only 1 small study has reported decreased CSF concentrations of 5-hydroxyindole acetic acid (serotonin metabolite) (Thimm *et al.,* 2011). Mechanisms that have been proposed suggest: (1) that tyrosine may inhibit tryptophan hydroxylase activity, which is the rate limiting step in serotonin metabolism and (2) tyrosine may compete for transport into the central nervous system via a common large neutral amino acid transporter (LAT-1) and thus reduce tryptophan uptake required for serotonin biosynthesis (Hillgartner *et al.,* 2016). It is reassuring that tryptophan did not change following nitisinone in the present data set.

Phenylalanine showed an increasing trend over the period studied. This is in contrast to reports in patients with HT1 that have shown reduced concentrations of phenylalanine following nitisinone therapy (Daly *et al.,* 2012; van Vliet *et al.,* 2014). While HT1 patients are on a protein restricted diet it is still thought that nitisinone itself may be the cause of the lower phenylalanine observed. The underlying mechanism for this is not fully understood (Harding *et al.,* 2014). It has been postulated that hypertyrosinaemia may also reduce phenylalanine uptake into the brain due to competition for the LAT-1 (van Ginkel *et al.,* 2016). This supposition may be supported as a significant increase in the ratio calculated in equation b (tyrosine to large neutral amino acids), and significant decrease in the ratios calculated in equations c-d (phenylalanine to LNAA; arginine to BCAA) were observed after nitisinone treatment implying that amino acid availability to the brain may be reduced, with the exception of tyrosine. The ratios are proposed to be a more reliable measure of the availability of tyrosine, phenylalanine and arginine to the brain instead of individual amino acid concentrations (Lieberman, 1999; Ravaglia *et al*., 2004).

While the significant decrease in the ratios implies a reduced availability of the detailed amino acids to the brain for neurotransmitter synthesis it does not fit with the serum concentrations of phenylalanine observed in the current

survey. However the concentration of phenylalanine in serum does not necessarily reflect that which is seen in CSF.

Of note pre-nitisinone therapy, the phenylalanine to tyrosine ratio was increased compared to the normal reference range (Antener *et al.,* 1981). The reason for this is uncertain, as typically this ratio is monitored to assess the flux of phenylalanine to tyrosine and the adequacy of protein intake. One may postulate that phenylalanine hydroxylase activity may be reduced, thus causing a mild increase in the ratio. Reduced enzyme activity has been reported in individuals with increased inflammation (Wannemacher *et al.,* 1976). As AKU is a chronic disease process it is possible that inflammatory processes are altered, however this was not assessed. Another possibility is that the increase in phenylalanine is an artefact; however this is unlikely as samples were centrifuged immediately and stored at -20 °C until they were analysed.

Following the commencement of nitisinone treatment the BCAA to AAA ratio significantly decreased, suggesting that following treatment liver function was altered. However this is potentially misleading as tyrosine is in such vast quantities in AKU following treatment with nitisinone and is not related to liver function; consequently one cannot use this ratio reliably to make an assessment of liver function. Prior to starting treatment the BCAA to AAA ratio was within the normal range indicating that patients had normal liver function.

A number of changes were observed in several amino acids over the course of this longitudinal survey, but none appear to be related to treatment with nitisinone and are not consistent at all-time points evaluated. It is thought that these findings are likely to reflect the natural fluctuation one would observe in amino acids, and no reliable conclusions can be drawn from these data.

Of particular interest were the mean amino acid concentrations (Table 4.1) that were outside the normal reference range independent of nitisinone

therapy. Specifically cystine was below the lower reference range and arginine and aspartic acid concentrations were higher.

Decreases in cystine can be observed due to haemolysis, delayed separation and or platelet/leukocyte contamination (Perry *et al.,* 1969; Bowron *et al.,* 2012). As discussed previously samples were separated immediately from cells and stored before analysis at -20 °C, thus eliminating this as a mechanism for the low cystine concentrations observed. One may postulate that the low cystine observed is a result of its reaction with glutathione, via glutathione-cystine transhydrogenase activity to form cysteine as part of the body's defence against oxidative stress. The rationale for this is that AKU is accompanied by increased oxidative stress (Davison *et al.,* 2016), which in part, results from the benzoquinone polymers that are produced from the high concentrations of HGA observed in AKU. Cysteine was not measured in this longitudinal survey, but should be evaluated to assess the validity of this hypothesis. The elevated arginine concentrations cannot be explained and require further investigation.

Decreases in asparagine and glutamine were observed following treatment with nitisinone. It has been proposed that increases in aspartic acid and glutamic acid can be observed from the deamination of asparagine and glutamine, respectively, due to delayed separation and haemolysis (Perry *et al.,* 1969, Bowron *et al.,* 2012). Herein samples did not visually appear to be haemolysed, however no haemolytic indices were measured so this cannot be completed excluded as a contributory factor. Moreover samples were separated immediately from cells and stored before analysis at -20 °C. One possibility for the decrease in glutamine is that the proposed increased oxidative stress observed in AKU results in the increased formation of glutamate (not measured) from glutamine. Glutamate is the precursor for glutathione, which is essential for maintaining the redox state of the cell (Davison *et al.,* 2016). Glutamic acid did not increase post nitisinone treatment and thus supports this supposition.

If deamination of asparagine to aspartic acid was the cause of increased aspartic acid in this survey one would have expected lower concentrations of asparagine to be observed, which was not the case. The significance of this finding is unknown.

4.5 Conclusions

Significant hypertyrosinaemia has been demonstrated following treatment with the HGA lowering drug nitisinone. The implications of the hypertyrosinaemia are in large unknown. However in this longitudinal survey it has been demonstrated that amino acid ratios have been significantly altered suggesting that the availability of amino acids for neurotransmitter biosynthesis, and liver function may be altered.

4.6 Declaration and acknowledgements

Preparation and analysis of serum samples for amino acids was performed by Andrew Davison and Elizabeth Smith (Department of Clinical Biochemistry, Alder Hey Children's Hospital, Liverpool, UK). All data analysis performed by Andrew Davison.

Jean Devine (Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Royal Liverpool Hospital, Liverpool, UK) processed all serum samples from patients attending the NAC.

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Chapter 5

Assessment of the effect of once daily nitisinone therapy on 24 hour urinary metadrenalines and 5-hydroxyindole acetic acid excretion in patients with alkaptonuria after 4 weeks of treatment

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5.1 Abstract

Background: One of the major metabolic consequences of using nitisinone to treat patients with Alkaptonuria (AKU) is that circulating tyrosine concentrations increase. As tyrosine is required for the biosynthesis of catecholamine neurotransmitters it is possible that their metabolism is altered as a consequence. Herein we report the 24 h urinary excretion of normetadrenaline (NMA), metadrenaline (MA), 3-methoxytyramine (3-MT) (catecholamine metabolites) and 5-hydroxyindole acetic acid (5-HIAA, metabolite of serotonin) in a cohort of AKU patients before and after 4 weeks of treatment with nitisinone.

Materials and methods: 24 h urinary excretion of NMA, MA, 3-MT, and 5-HIAA were determined by liquid chromatography tandem mass spectrometry. Inter-assay coefficient of variation was <10 % for all analytes measured, at all concentrations tested.

Results: Urine samples were assayed at baseline (pre-nitisinone, n=36) and 4 weeks later; 7 received no nitisinone (4 male, mean age (±SD) 46.3 (16.4) years) and 29 received a daily dose of nitisinone [1 mg (n=7, 6 male, mean age 45.9 (10.9) years), 2 mg (n=8, 5 male, mean age 43.9 (13.7) years), 4 mg (n=8, 5 male, mean age 47.3 (10.7) years) and 8 mg (n=6, 4 male, mean age 53.8 (8.3) years)]. 3-MT concentrations increase significantly ($p<0.01$, at all doses) following nitisinone therapy, but not in a dose dependent manner. NMA concentrations decreased ($p<0.05$, at all doses) following nitisinone therapy at all doses. 5-HIAA concentrations decreased following nitisinone therapy, and were significantly lower at a daily dose of 8 mg only ($p<0.05$).

Conclusions: This study shows that catecholamine and serotonin metabolism is altered by treatment with nitisinone.

5.2 Introduction

Alkaptonuria (AKU, OMIM 203500) is a rare autosomal recessive disorder of tyrosine metabolism (Figure 5.1 (A)) resulting from a congenital lack in the enzyme homogentisate-1,2-dioxygenase (HGD, E.C.1.12.11.5). It occurs in 1 in 250 000 of the general population (Phornphutkul *et al.,* 2002), however in certain countries it is observed more commonly; for instance in Slovakia it is estimated to occur in 1 in 19 000 of the population (Milch, 1960; Zatkova, 2011). One of the major biochemical consequences of AKU is that the circulating concentration of homogentisic acid (HGA) significantly increases. HGA is central to the pathophysiology of the disease and is thought to be responsible for a number of abnormalities including spondyloarthropathy, characterised by progressive kyphoscoliosis and impaired spinal and thoracic mobility, as well as renal and prostate stones, aortic valve stenosis, osteoporosis, fractures, and ruptures of tendons, ligaments and muscle (Ranganath *et al.,* 2013).

Supportive medical management of AKU includes a low protein diet, analgesia and arthroplasty (Ranganath *et al.,* 2013). An additional HGA lowering therapy is nitisinone (Figure 5.1 (A)), a competitive reversible inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C. 1.13.11.27). Its action reduces the accumulation of HGA and thus has the potential to prevent or slow the complications observed in patients with AKU. Currently nitisinone is not licenced for the treatment of patients with AKU.

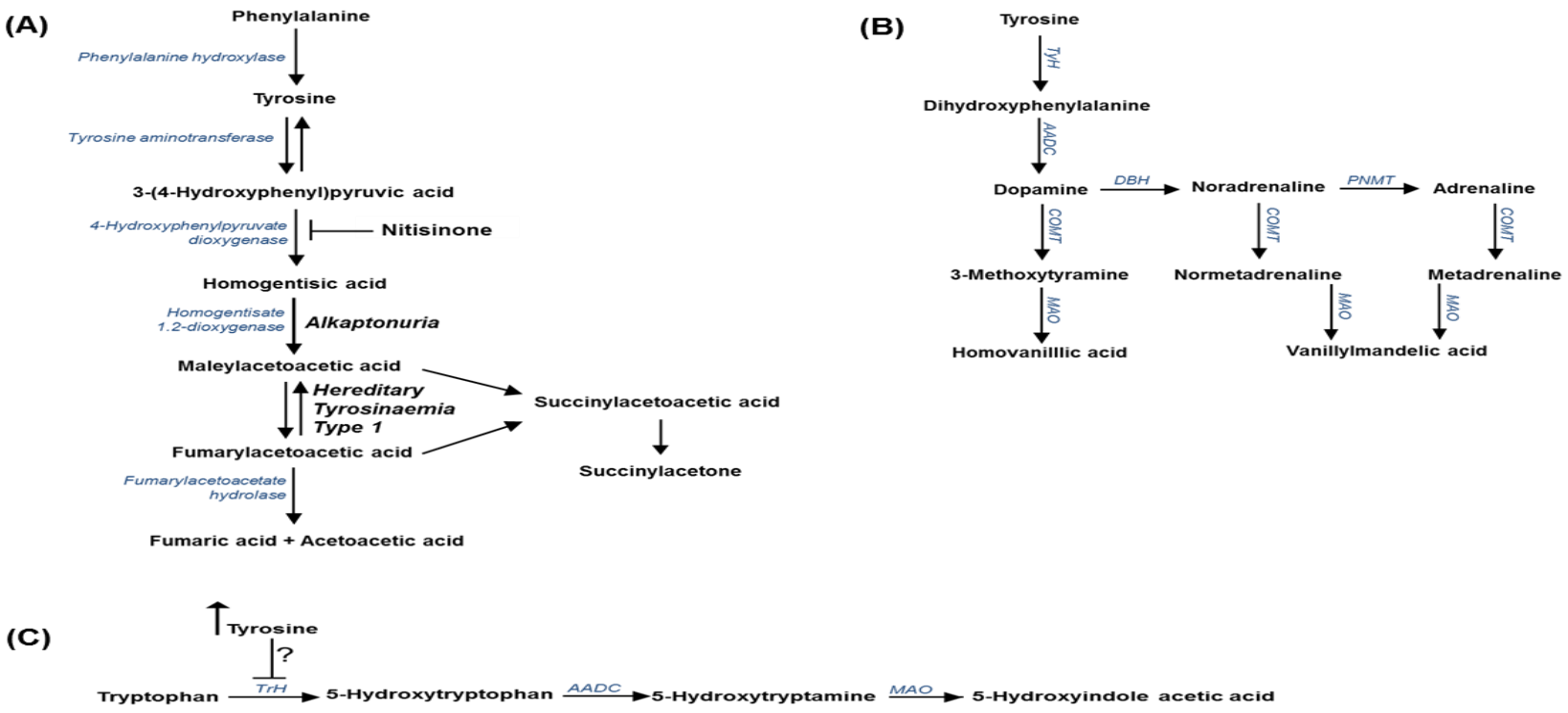

Figure 5.1. (A) Tyrosine metabolic pathway – highlighting the site of the enzyme defect observed in alkaptonuria and hereditary tyrosinaemia type 1, and the site of action of nitisinone; (B) Catecholamine metabolic pathway showing the formation of metadrenalines and (C) Tryptophan metabolic pathway – highlighting the proposed site tyrosine inhibits tryptophan hydroxylase activity. TyH – tyrosine hydroxylase; AADC – aromatic acid decarboxylase; COMT – catechol-O-methyl transferase; MAO – monoamine oxidase; TPH – tryptophan hydroxylase.

In contrast nitisinone is already licenced for the treatment of hereditary tyrosinaemia type 1 (HT1, OMIM 276700) and has proved to be a very efficacious mode of treatment (McKiernan, 2013).

One of the major metabolic consequences of treating patients with nitisinone in AKU and HT1 is that circulating tyrosine concentrations increase significantly (Lindstedt *et al.,* 1992; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; McKiernan *et al.,* 2015; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017). As tyrosine is the metabolic substrate required for the biosynthesis of catecholamine neurotransmitters (adrenaline (Ad), noradrenaline (NA) and dopamine (DP)) (Figure 5.1 (B)) it is possible that they may be altered during the 4 week treatment trial with nitisinone.

Hypertyrosinaemia is observed in patients with AKU that attend the National AKU Centre in Liverpool (patients are given 2 mg of nitisinone daily, off-licence) and during the Suitability Of Nitisinone In Alkaptonuria 1 (SONIA-1) clinical trial (Ranganath *et al.,* 2016), which evaluated the efficacy of different daily doses of nitisinone over a 4 week period.

Significant elevations in serum and cerebrospinal fluid tyrosine concentrations have also been a documented consequence of nitisinone treatment in HT1 (Thimm *et al.,* 2011). In patients with HT1 it is believed that the supraphysiological concentrations of tyrosine may be responsible for the reduced intelligence quotient and cognitive function observed (Masurel-Paulet *et al.,* 2008; De Laet *et al.,* 2011; Thimm *et al.,* 2012; Bendadi *et al.,* 2014; Mckiernan *et al.,* 2015).

The impact of hypertyrosinaemia on neurotransmitter metabolism (Figures 5.1 (B) and (C)) in patients with AKU following nitisinone therapy has not been previously reported. Herein we report the 24 h urinary excretion of normetadrenaline (NMA), metadrenaline (MA) and 3-methoxytyramine (3-MT) (catecholamine metabolites) and 5-hydroxyindole acetic acid (5-HIAA) (metabolite of serotonin) in patients before and during the 4 week treatment trial with nitisinone.

5.3 Materials and methods

5.3.1 Subjects

All urine samples were from subjects included in the SONIA-1 clinical trial (Trial registration number EudraCT number: 2012-005340-24. Registered at ClinicalTrials.gov: NCTO1828463). 24 h urine samples were collected into 2.5 L bottles containing 30 mL of 5N H_2SO_4; aliquots were stored away from bright light at -80 °C. Urine samples analysed in this study were from baseline (pre-nitisinone, n=36) and 4 weeks following no treatment (n=7) or a daily dose of nitisinone [1 mg (n=7), 2 mg (n=8), 4 mg (n=8) and 8 mg (n=6)]. No patients included in this study had renal impairment (eGFR >60 mL/min/1.73 m^2 in all cases). Completeness of 24 h urine collection was assessed by measurement of urine creatinine (Jaffe reaction, Roche Diagnostics, Germany) all patients had urine creatinine concentrations within the normal reference range (9.0-18.0 μmol/24 h, in house reference range).

5.3.2 Analytical methods

5.3.2.1 Urine metadrenalines

The concentrations of urinary NMA, MA and 3-MT were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Banks *et al.,* 2014). 1 mL of urine underwent acid hydrolysis (5 M HCl) at 100 °C for 30 min. 50 μL of hydrolysate was diluted in 2 mL of deionised water containing deuterated internal standards (d_3-NMA, 0.11 μmol/L, d_3-MA 0.10 μmol/L and d_4-3-MT 0.12 μmol/L. CDN Isotopes, Essex) and loaded onto the solid phase extraction plate (30 mg Evolute-SCX, Biotage, Hengoed). 20 μL of extract was injected onto a C_{18} phenyl-hexyl column (4.6 x 100 mm, 2.6 μm, Phenomenex, Cheshire) using a Waters Acquity UPLC separations module coupled to a Xevo TQS tandem mass spectrometer. Initial conditions of 90:10 water:methanol with 0.1 % formic acid (v/v) increased linearly to 40:60 over 2 min, returning to starting conditions by 4 min. In-house calibration standards and commercial quality controls materials (Recipe ClinChek, Germany) were used. Calibrator concentrations were 0-46.8 μmol/L for NMA; 0-24.8 μmol/L for MA and 0-27.7 μmol/L for 3-MT. Interassay coefficient of

variation for NMA, MA and 3-MT were 4.6, 4.7 and 7.2 % at 1.7, 0.8 and 1.6 μmol/L, respectively.

5.3.2.2 Urine 5-HIAA

The concentration of urinary 5-HIAA was determined by LC-MS/MS. Samples were diluted (20 μL urine in 2 mL) in deionised water containing a deuterated internal standard (d_5-5-HIAA, 0.34 μmol/L, CDN Isotopes, Essex). 20 μL of the diluted urine sample was injected onto an Atlantis dC_{18} column (3.0 x 100 mm, 3.0 μm, Waters, Milford) using a Waters Alliance 2795 separations module coupled to a Waters Quattro Premier XE tandem mass spectrometer. Initial conditions of 95:5 water:methanol with 0.1 % formic acid (v/v) and 2 mmol/L ammonium acetate, increased linearly to 5:95 over 2 min, returning to starting conditions by 4 min. Commercial calibration standards (3.6-356 μmol/L, Chromsystems, Germany) and quality controls materials (Recipe ClinChek, Germany) were used. Interassay coefficient of variation was 7.2 % for 5-HIAA at 26.2 μmol/L.

5.3.2.3 Serum tyrosine and phenylalanine

The concentration of serum tyrosine and phenylalanine were determined by LC-MS/MS, for details see Hughes *et al.* (2015). Tyrosine concentrations were previously reported in the SONIA-1 clinical trial (Ranganath *et al.,* 2016).

In this study data from only 36 of 40 subjects from the SONIA-1 clinical were included. Serum tyrosine and phenylalanine concentrations presented herein are from subjects that had urine samples analysed for urinary NMA, MA, 3-MT and 5-HIAA. All serum samples were collected from patients after an overnight fast (at least 8 h). Patients' dietary intake of protein was not restricted during this study, nor was it monitored.

5.3.3 Statistical Analysis

All statistical analysis were performed using Graphpad Instat (Version 3.10, 2009). Kolmogorov-Smirnov testing was performed to assess if urinary NMA, MA, 3-MT and 5-HIAA concentrations and serum tyrosine concentrations

were normally distributed. Unpaired 2-tailed student t-test was used to assess significant differences in urinary metabolites pre- and post-treatment with nitisinone, a p value <0.05 was deemed significant.

5.4 Results

Forty patients were included in the SONIA-1 study (Ranganath *et al.,* 2016). Urine samples from 36 of these patients were available for inclusion in this study. Of the 36 patients 7 patients received no treatment with nitisinone (4 male, mean age (±SD) 46.3 (16.4) years) and 29 patients received a daily dose of nitisinone [1 mg (n=7, 6 male, mean age 45.9 (10.9) years), 2 mg (n=8, 5 male, mean age 43.9 (13.7) years), 4 mg (n=8, 5 male, mean age 47.3 (10.7) years) and 8 mg (n=6, 4 male, mean age 53.8 (8.3) years)].

Urinary 3-MT concentrations increased significantly ($p \leq 0.01$, at all doses) following daily nitisinone therapy. This occurred at all doses, but not in a dose dependent manner. In contrast urinary NMA concentrations decreased ($p \leq 0.03$, at all doses) following nitisinone therapy. Urinary MA concentrations were not significantly different post nitisinone therapy (Figure 5.2).

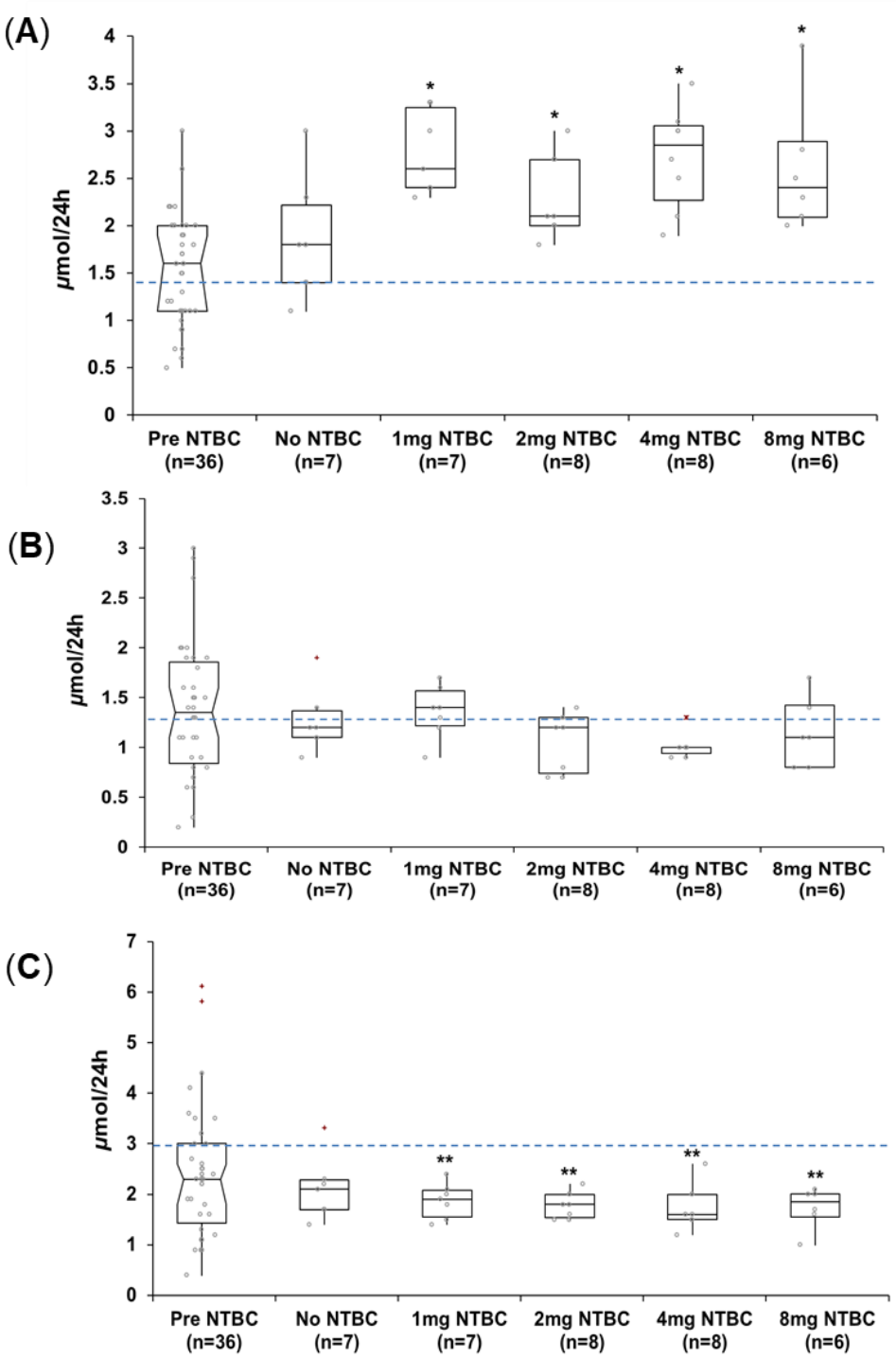

Figure 5.2. Urinary metadrenaline concentrations in patients with AKU before and after 4 weeks of treatment with nitisinone. At 4 weeks there were 5 treatment groups – no treatment (n=7); treatment with 1 mg nitisinone daily (n=7); treatment with 2 mg nitisinone daily (n=8); treatment with 4 mg nitisinone daily (n=8) and treatment with 8 mg nitisinone daily (n=6). (A) 3-MT; (B) MA and (C) NMA. --- = indicates upper limit of normal urine reference

range for 3-MT <1.4 μmol/24h; MA <1.3 μmol/24h; NMA <3.0 μmol/24h; - = 95 % confidence notched outlier boxplot; + = outlier; o=individual patient concentrations. Significance testing compared metadrenalines before and after a 4 week trial of nitisinone, *p≤0.01; **p≤0.03; p value <0.05 deemed significant. NTBC – nitisinone.

Of interest a large proportion of patients had NMA (reference range <3.0 μmol/24 h), MA (reference range <1.3 μmol/24 h) and 3-MT (reference range <1.4 μmol/24 h) concentrations outside of the normal reference range pre-nitisinone therapy. Following nitisinone therapy NMA concentrations were within the normal reference range in all but 1 patient, however 3-MT concentrations were outside of the reference range in all patients. MA concentrations remained unchanged overall post nitisinone therapy, although there was less of a spread of concentrations observed.

5-HIAA concentrations decreased following nitisinone therapy (Figure 5.3), and were significantly lower at a daily dose of 8 mg ($p<0.05$) only. 5-HIAA concentrations were within the normal reference range (reference range <50 μmol/24 h) in the majority of patients pre- (7/36 patients had elevated 5-HIAA) and post (2/8 patients receiving no nitisinone and 1/7 patients receiving 1 mg nitisinone daily had elevated 5-HIAA) nitisinone therapy.

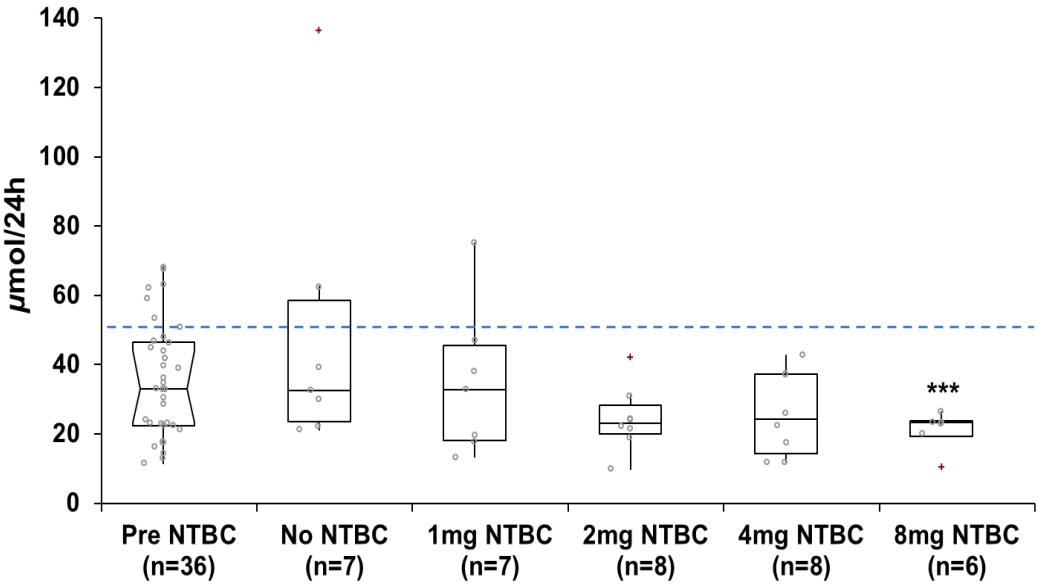

Figure 5.3. Urinary 5-HIAA concentrations in patients with AKU before and after 4 weeks of treatment with nitisinone. At 4 weeks there were 5 treatment groups – no treatment (n=7); treatment with 1 mg nitisinone daily (n=7); treatment with 2 mg nitisinone daily (n=8); treatment with 4 mg nitisinone daily (n=8) and treatment with 8 mg nitisinone daily (n=6).--- = indicates upper limit of normal urine reference range for 5-HIAA, <50 µmol/24h; - = 95 % confidence notched outlier boxplot; + = outlier; o=individual patient concentrations. Significance testing compared 5-HIAA concentrations before and after a 4 week trial of nitisinone, ***p<0.05. p value <0.05 deemed significant. NTBC – nitisinone.

Fasting serum tyrosine concentrations increased significantly following treatment with nitisinone at all doses (p<0.0001). There were also significantly higher tyrosine concentrations observed between subjects receiving 1 mg nitisinone versus 8 mg nitisinone (p<0.05) and subjects receiving 2 mg nitisinone versus 8mg nitisinone (p<0.05), thus indicating a dose dependent increase in serum tyrosine concentrations following nitisinone. Mean (± standard deviation) concentrations were 59 (±10) µmol/L and 750 (±140) µmol/L pre- and post-nitisinone therapy (Figure 5.4), respectively (reference range 30-87 µmol/L (Davison *et al.,* 2015)).

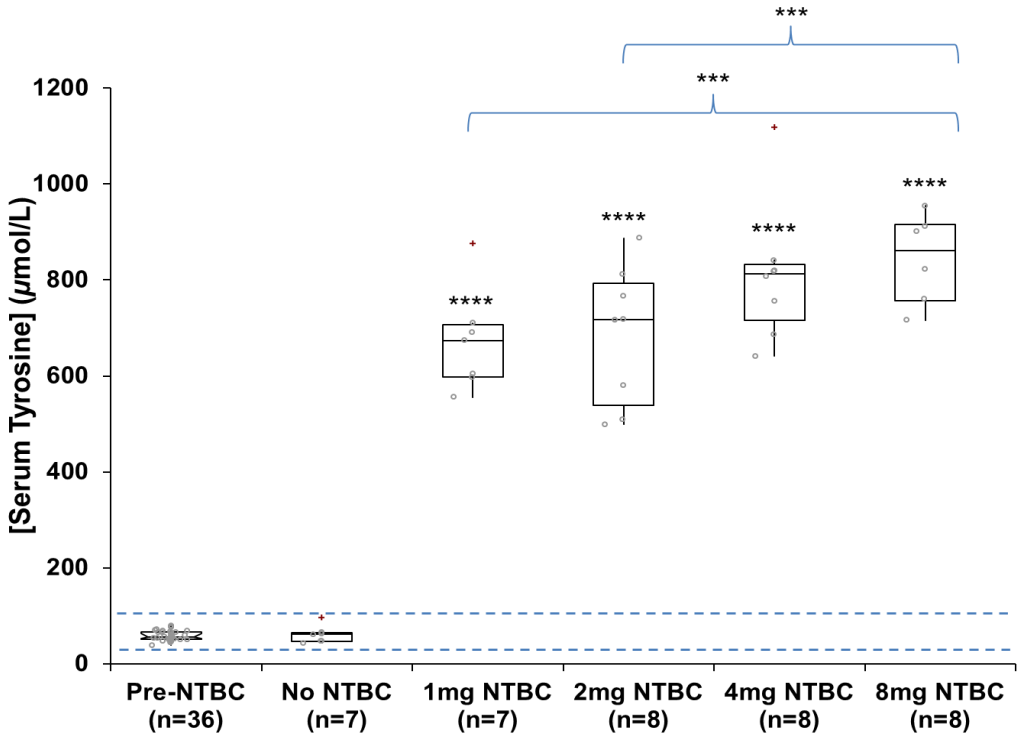

Figure 5.4. Serum tyrosine concentrations in patients with AKU before and after 4 weeks of treatment with nitisinone. At 4 weeks there were 5 treatment groups – no treatment (n=7); treatment with 1 mg nitisinone daily (n=7); treatment with 2 mg nitisinone daily (n=8); treatment with 4 mg nitisinone daily (n=8) and treatment with 8 mg nitisinone daily (n=6). --- = indicates normal serum reference range for tyrosine, 30-87 µmol/L; - = 95 % confidence notched outlier boxplot; + = outlier; o=individual patient concentrations. Significance testing compared tyrosine concentrations before and after a 4 week trial of nitisinone, and tyrosine concentrations in patients that received different doses of nitisinone at 4 weeks of therapy (brackets compare 1 mg vs. 8 mg nitisinone, and 2 mg vs. 8 mg nitisinone), **** p<0.0001; ***p<0.05. p value <0.05 deemed significant. NTBC – nitisinone.

Serum phenylalanine concentrations were not significantly different following a 4 week trial of nitisinone (p>0.05 at all doses) and were within the normal reference range (30-76 µmol/L, in-house reference range). Mean (±standard deviation) phenylalanine concentrations were 57.1 µmol/L (7.9) (pre-nitisinone, n=36); 55.7 (17.8) µmol/L (no nitisinone, n=7); 53.0 (6.0) µmol/L (1

mg nitisinone, n=7); 51.6 (13.0) μmol/L (2 mg nitisinone, n=8); 53.8 (12.2) μmol/L (4 mg nitisinone, n=8) and 53.8 (13.4) μmol/L (8 mg nitisinone, n=6).

5.5 Discussion

Significant hypertyrosinaemia following nitisinone therapy has been previously reported in AKU (Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017). To date there are no reports on the impact this may have on closely related metabolic pathways that require tyrosine as a substrate, including the biosynthesis of the catecholamine neurotransmitters (Figure 5.1 (B)).

Much of the literature focuses on hypertyrosinaemia following nitisinone therapy in patients with HT1. It is believed that the supraphysiological concentrations observed may contribute to neurodevelopmental delay (Masurel-Paulet *et al.,* 2008; De Laet *et al.,* 2011; Thimm *et al.,* 2012; Bendadi *et al.,* 2014; Mckiernan *et al.,* 2015). It is estimated that up to 35 % of children treated with nitisinone have learning difficulties (McKiernan *et al.,* 2015). It is not known whether this may also be related to nitisinone treatment itself or low phenylalanine concentrations, presenting with acute liver disease or an intrinsic effect of HT1.

Several mechanisms for neurodevelopmental delay have been postulated. These include: increased transport of tyrosine into the brain; decreased transport of other neutral amino acids into the brain (specifically tryptophan, the precursor of serotonin); increased central nervous system (CNS) DP; decreased CNS serotonin, oxidative damage from δ-aminolevulinic acid and succinylacetone modification of neuronal proteins (Thimm *et al.,* 2011; Harding *et al.,* 2014; Hillgartner *et al.,* 2016).

It has also been suggested that altered serotonin metabolism may be due to direct inhibition of tryptophan hydroxylase (TPH, EC 1.14.16.4) activity by tyrosine (Figure 5.1 (C)), which leads to a reduced biosynthesis of serotonin

(Thimm *et al.,* 2011). TPH is the rate limiting step in the biosynthesis of serotonin.

As it has been suggested that hypertyrosinaemia may have an impact on neurodevelopmental delay in HT1 concern exists around the use of nitisinone in AKU, which is currently not licenced for treatment of this disorder. While the dose prescribed to patients with AKU is much lower than those with HT1 (2 mg daily at the National Centre for AKU (NAC) in Liverpool versus 0.5-2.5 mg/kg per day in HT1) the concentration of serum and urine tyrosine observed following nitisinone therapy is similar.

Herein for the first time we report the impact of nitisinone on the urinary excretion of the NMA, MA and 3-MT, and 5-HIAA, which serve as surrogate markers for catecholamine and serotonin neurotransmitter biosynthesis, respectively (Figures 5.1 (B) and (C)). Urinary metabolites were measured as collecting urine is non-invasive and is the primary route for neurotransmitter elimination.

The marked increase in 3-MT and decreased NMA excretion post nitisinone therapy indicates that nitisinone therapy alters the metabolism of catecholamines as the concentration of their respective O-methylated metabolites are altered when compared to pre-treatment concentrations.

The metabolism of catecholamine neurotransmitters is complex (Figure 5.1 (B)) and they have multiple origins, including the sympathetic nerves, adrenal medulla, brain and mesenteric organs (for detailed overview see Eisenhofer *et al.,* 2004). It has been demonstrated that the concentration of monoamine neurotransmitters in urine are dependent on plasma concentration and uptake via organic cation transporters in the kidney (Eisenhofer *et al.,* 1996; Graefe *et al.,* 1997). However it is also essential to consider the degree to which renal neurotransmitter synthesis can contribute to the urinary concentrations. In the kidneys DP is produced via the uptake and decarboxylation of circulating dihydroxyphenylalanine (DOPA), and not just from the filtration of circulating DP (Eisenhofer *et al.,* 2004).

In this study 3-MT was shown to increase significantly post nitisinone therapy. 3-MT is a direct metabolite of DP and thus may reflect increased circulating DP concentrations and or synthesis in the kidney. It is proposed that the latter is highly likely as the tyrosine load delivered to the kidney increased significantly post nitisinone therapy. This was reported in the SONIA-1 dosing study (Ranganath *et al.,* 2016) and shown in the data presented herein where mean serum tyrosine concentrations increased significantly ($p<0.0001$) after a 4 week treatment with nitisinone therapy (reference range 30-87 μmol/L, Davison *et al.,* 2015). The increased tyrosine load delivered to the kidney may provide a substrate for DOPA synthesis and thus the subsequent decarboxylation of DOPA to DP may lead to the consequent increase in 3-MT observed.

The elevated serum tyrosine concentrations observed post nitisinone therapy was accompanied by a significant dose dependent decrease in urinary excretion of HGA, across the studied dose interval of 1–8 mg. The 8 mg dose resulted in a mean reduction of 24 h urinary HGA excretion of
98.8 % compared with baseline (Ranganath *et al.,* 2016). Serum phenylalanine concentrations were not affected by the marked increase in tyrosine following a 4 week trial with nitisinone, indicating that tyrosine has an alternative metabolic fate.

The impact of hypertyrosinaemia on serum tryptophan concentrations were not evaluated in this short term dose evaluation study as it was not included in the original study design. However it has been shown at the NAC in Liverpool that serum tryptophan concentrations do not change following a 2 mg daily dose of nitisinone (Davison *et al.,* 2018a). The authors postulate that serum tryptophan concentrations will not be altered at a higher dose of nitisinone as marked hypertyrosinaemia is observed at all doses of nitisinone evaluated. Further work is required to confirm this.

In this study tissue concentrations of tyrosine were not determined to assess whether they correlate with serum and urine concentrations. Further work

into this area is required to help understand the pathophysiology of AKU at a tissue level.

Previously Thimm *et al.* (2011) reported tyrosine and homovanillic acid (dopamine metabolite) concentrations in the CSF of 3 patients with HT1 during long-term treatment with nitisinone. In this small study tyrosine concentrations were markedly increased in the CSF and plasma, as expected. However homovanillic acid concentrations were within the normal reference range, suggesting that there is no alteration in DP metabolism in the CNS. This supports the postulate that the increased urinary 3-MT observed in this study is a consequence of increased tyrosine load being delivered to the kidney. It is also important to consider that dietary constituents have been shown to influence urinary concentrations of 3-MT and thus represent 1 peripheral source of DP metabolites (de Jong, 2009).

In this study dietary components rich in biogenic amines were not restricted or documented. However as the data presented herein show an increase in 3-MT in all patients following a 4 week treatment with nitisinone it is believed that diet alone is not solely responsible for the increase in 3-MT observed.

The parallel decrease in NMA suggests that there may be a reduction in the synthesis of NA as a consequence of nitisinone therapy. While there is limited information available on the exact contribution of central and peripheral output to urinary excretion of neurotransmitters Graefe *et al.* (1997) demonstrated that a significant proportion of urinary NA and Ad stems from circulation. In this study one may postulate the observed decrease in NMA may be a consequence of reduced sympathetic nerve excitation as subjects are less depressed or anxious, as they are on treatment for AKU. Previous studies (Roy *et al.,*1986a; Roy *et al.,* 1986b; Grossman *et al.,* 1999; Hughes *et al*., 2004) have evaluated the urinary concentration of NA and Ad in subjects with depression, demonstrating that urinary concentrations of NA and Ad were significantly higher in subjects with depression compared to controls. It is not surprising that MA concentrations were not significantly different post nitisinone therapy as Ad is produced by the adrenal gland and

not the sympathetic nervous system, mesenteric organs or the kidney (Eisenhofer, 2004). The reduced variability of MA concentrations observed following a 4 week trial of nitisinone is of unknown significance and is unlikely to reflect a change in diet, this requires further investigation.

Another possibility is that there is an alteration in inflammatory signalling following nitisinone therapy. Elenkov *et al.* (2000) demonstrated that Ad and NA can inhibit the production of pro-inflammatory cytokines (*i.e.* interleukin-2, tumour necrosis factor-α, and interferon-γ) and stimulate the production of anti-inflammatory cytokines (*i.e.* interleukin-10 and transforming growth factor-β). It is postulated that altering these pathways may cause a selective suppression of T helper-1 cells and cellular immunity, and enhancement of T helper-2 cell activity and a shift toward humoral immunity.

While changes in urinary concentrations of NA, Ad and their respective metabolites were not evaluated in this study, one can postulate that they may be altered as a consequence of changes in the inflammatory processes observed following nitisinone therapy.

The decrease in 5-HIAA excretion at a daily dose of 8 mg only, suggests that there is a dose dependent effect of nitisinone therapy. This supports that serotonin metabolism is altered following treatment with nitisinone. Thimm *et al.* (2011) also reported alterations in serotonin metabolism in patients with HT1 treated with nitisinone. This study showed a decrease in CSF 5-HIAA concentrations following nitisinone therapy.

It is proposed that the decrease in CSF 5-HIAA occurred as increased tyrosine concentrations compete with tryptophan (serotonin precursor) via a neutral amino acid transporter across the blood-brain barrier, thus reducing tryptophan availability for intracerebral serotonin synthesis (Pratt *et al.,* 1982).

In addition it has been hypothesised that elevated tyrosine may inhibit tryptophan hydroxylase activity (Figure 5.2 (C)), the rate limiting step for

serotonin metabolism (Thimm *et al.,* 2011). Although this study was small and analysis was performed in CSF it has been shown that urinary analysis of serotonin and 5-HIAA reflect parallel changes in immunoreactivity in the dorsal raphe nucleus, demonstrating a positive correlation between CNS serotonergic activity and urinary serotonin concentrations (Lynn-Bullock *et al.,* 2004).

5.6 Conclusion

For the first time alterations in monoamine neurotransmitter metabolism have been reported in patients with AKU following nitisinone therapy. Specifically increased urinary 3-MT (dopaminergic neurotransmitter metabolite), and decreased NMA (NA neurotransmitter metabolite) and 5-HIAA (serotoninergic neurotransmitter metabolite) concentrations. The exact mechanism(s) causing these changes are not known and further work is required to elucidate this, and to establish whether these observations truly reflect changes in CNS monoamine neurotransmitter metabolism or contributions from peripheral or renal metabolism.

Moreover it is recognised that these data are based on a short term dosing study and it is necessary to assess whether these changes would be observed in patients on long term therapy with nitisinone and to see if biochemical changes correlate with changes in behaviour or mood.

5.7 Declaration and acknowledgements

Preparation, analysis and data processing associated urine samples for the measurement of metadrenalines and 5-HIAA performed by Andrew Davison.

Jean Devine and Jeanette Usher (Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Royal Liverpool Hospital, Liverpool, UK) processed all urine samples from patients recruited into the SONIA-1 clinical trial.

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Chapter 6

Clinical and biochemical assessment of depressive symptoms in patients with alkaptonuria before and after 2 years of treatment with nitisinone

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6.1 Abstract

Background: Concerns exist over hypertyrosinaemia that is observed following treatment with nitisinone. It has been suggested that tyrosine may compete with tryptophan for uptake into the central nervous system, and or inhibit tryptophan hydroxylase activity reducing serotonin production. At the National Alkaptonuria (AKU) Centre nitisinone is being used off-licence to treat AKU, and there is uncertainty over whether hypertyrosinaemia may alter mood. Herein results from clinical and biochemical assessments of depression in patients with AKU before and after treatment with nitisinone are presented.

Materials and methods: 63 patients were included pre-nitisinone treatment, of these 39 and 32 patients were followed up 12 and 24 months after treatment. All patients had Beck's Depression Inventory-II (BDI-II) assessments (scores can range from 0-63, the higher the score the more severe the category of depression), and where possible urinary monoamine neurotransmitter metabolites and serum aromatic amino acids were measured as biochemical markers of depression.

Results: Mean (±standard deviation) BDI-II scores pre-nitisinone, and after 12 and 24 months were 10.1(9.6); 9.8(10.0) and 10.5(9.9) ($p>0.05$, all visits). Paired scores (n=32), showed a significant increase at 24 months compared to baseline 10.5(9.9) vs. 8.6 (7.8) ($p=0.03$). Serum tyrosine increased at least 6-fold following nitisinone ($p<0.0001$, all visits), and urinary 3-methoxytyramine (3-MT) increased at 12 and 24 months ($p<0.0001$), and 5-hydroxyindole acetic acid (5-HIAA) decreased at 12 months ($p=0.03$).

Conclusions: BDI-II scores were significantly higher following 24 months of nitisinone therapy in patients that were followed up, however the majority of these patients remained in the minimal category of depression. Serum tyrosine and urinary 3-MT increased significantly following treatment with nitisinone. In contrast urinary 5-HIAA did not decrease consistently over the same period studied. Together these findings suggest nitisinone does not cause depression despite some observed effects on monoamine neurotransmitter metabolism.

6.2 Introduction

Alkaptonuria (AKU, OMIM 203500) is a rare autosomal recessive disorder of the tyrosine metabolic pathway, occurring 1 in 250 000 of the general population (Phornphutkul *et al.,* 2002). It results from a congenital deficiency in the enzyme homogentisate 1,2-dioxygenase (HGD, E.C.1.12.11.5). One of the major biochemical consequences of AKU is that the circulating concentration of homogentisic acid (HGA) significantly increases despite significant renal excretion. It is proposed that circulating HGA is responsible for a number of the complications which are observed, for a detailed review see Ranganath *et al.* (2013). The psychological impact of this painful and debilitating musculoskeletal disease has never been reported. Katon *et al.* (2002) reported that patients with chronic medical illness, such as diabetes, stroke, cancer and heart disease have a high prevalence of major depressive illness.

Assessing patients' psychological state before and after treatment is important as judgments can be made about whether treatment will result in a change in mood or depression. This is highly relevant when considering patients with AKU that are being treated with nitisinone, a competitive inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C. 1.13.11.27). This is because it results in marked hypertyrosinaemia (Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a). This has also been reported in patients with hereditary tyrosinaemia type 1 (HT1, OMIM 276700) treated with nitisinone (Lindstedt *et al.,* 1992; McKiernan *et al.,* 2013; McKiernan *et al.,* 2015; van Ginkel *et al.,* 2016; van Ginkel *et al.,* 2017).

The metabolic fate of these supraphysiological tyrosine concentrations is unknown. It has been reported that it can cause corneal keratopathy (Khedr *et al.,* 2018). In addition it is known that tyrosine is the precursor for the biosynthesis of the neurotransmitter dopamine (DP), which plays a role in mood.

In HT1 it is estimated that 35 % of children have neurodevelopmental delay (McKiernan *et al.,* 2015). Several mechanisms have been postulated, including: increased transport of tyrosine into the brain; decreased transport of other neutral amino acids into the brain (specifically tryptophan, the precursor of serotonin); increased central nervous system (CNS) DP; decreased CNS serotonin, oxidative damage from δ-aminolevulinic acid and succinylacetone (the toxic metabolites in HT1) or modification of neuronal proteins (Thimm *et al.,* 2011; Harding *et al.,* 2014; Hillgartner *et al.,* 2016). It has also been suggested that altered serotonin metabolism may be due to direct inhibition of tryptophan hydroxylase (TPH; EC 1.14.16.4) activity by tyrosine, which leads to a reduced biosynthesis of serotonin (Thimm *et al.,* 2011).

Due to the possibility that hypertyrosinaemia may have an impact on neurodevelopment in HT1, concerns exist around its off licence use in patients with AKU. Therapy is currently commenced when patients are ≥16 years old so they are less likely to suffer the same neurodevelopmental delay. Therefore the focus is on whether changes in neurotransmitter metabolism may affect mood and result in depression.

The impact of hypertyrosinaemia on neurotransmitter metabolism in patients with AKU following nitisinone therapy has been previously reported (Davison *et al.,* 2018b). Catecholamine neurotransmitter metabolites 3-methoxytyramine (3-MT, DP metabolite) and normetadrenaline (NMA) (noradrenaline (NA) metabolite) were shown to increase and decrease significantly following nitisinone therapy, respectively. The urinary serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) was shown to decrease following nitisinone therapy, but the decrease was only significant at the highest daily dose of 8 mg. These biochemical data were however limited in that this was a short term dosing study that did not report any psychometric data, and serum tryptophan and phenylalanine concentrations were not reported.

Herein for the first time we report BDI-II scores as a self-reporting measure of depression (Beck *et al.,* 1961; Beck *et al.,* 1996) in patients with AKU, pre-nitisinone and at 12 and 24 months of nitisinone therapy. In addition urinary monoamine metabolites of catecholamine and serotonin neurotransmitters and associated serum aromatic amino acids tyrosine, phenylalanine and tryptophan are reported in a sub-group of patients.

6.3 Materials and methods

6.3.1 Patients

6.3.1.1 Protocol for patients that attend the National Alkaptonuria Centre (NAC) for treatment with nitisinone

The protocol for treatment at the NAC is that patients with confirmed AKU are commenced on a 2 mg dose of nitisinone, on alternative days for the first 3 months, which is then increased to 2 mg daily thereafter. Assessments are repeated on an annual basis to monitor response to therapy.

Inclusion criteria for treatment with nitisinone are that individuals must have the diagnosis of AKU; must be a resident of England or Scotland, and be over the age of 16 years. Confirmed diagnosis of AKU is based upon increased urinary HGA excretion [urine HGA in healthy volunteers has been demonstrated in the order of <2.92 *μ*mol/day (Davison *et al.,* 2015)] and mandatory for referral to the NAC. Exclusion criteria are individuals must not be pregnant and or lactating. Nitisinone is used in an off-licence setting to investigate its safety and efficacy in the treatment of this rare disease. All patients are provided with written information about the scope of the Centre and the assessments they will receive. All patients at the NAC have biochemical measurements and clinical assessments which are performed at baseline, day 4 (2 days post-nitisinone), 3 months, 6 months and 12 months; with annual monitoring thereafter.

6.3.1.2 Ethical Approval

Data collection and analyses at the NAC has approval from the Royal Liverpool and Broadgreen University Hospital Trusts Audit Committee (Audit no. ACO3836). As data were collected as part of the clinical service ethical approval was not required. Data is obtained following standard clinical assessments upon referral to the NAC. Patients are informed verbally and through patient information leaflets about the activities of the NAC. Patients are also explicitly informed that data may be used for publication and within the NAC patient information leaflet.

6.3.1.3 Subjects included in the study

Sixty-three patients [26 female, mean age (±standard deviation) 51.3(16.8) years (range 18-75); 37 male, mean age 47.6(13.9) years (range 16-70)] were included at baseline in this 24 month longitudinal survey reporting the BDI-II data.

BDI-II data were also included at 12 (n=39) and 24 (n=32) months following 2 mg daily nitisinone. Twenty-four and 31 of the 63 patients included at baseline were not included at 12 and 24 months, respectively. Five patients were excluded as they were not resident of England or Scotland. The remaining 19 and 26 patients did not have follow-up visits at 12 and 24 months respectively, either because they did not attend or because they had not attended the NAC for long enough for follow-up visits at the point of data collection.

24 h urine samples for measurement of monoamine metabolites were collected into 2.5 L bottles containing 30 mL of 5 N H_2SO_4; aliquots were stored away from bright light at -20 °C. Urine samples were from baseline (pre-nitisinone), 3 months (2 mg nitisinone every other day), 6 months (2 mg nitisinone daily), 12 months (2 mg nitisinone daily) and 24 months (2 mg nitisinone daily). No patients included in this study had renal impairment (eGFR >60 mL/min/1.73 m^2 in all cases). Completeness of 24 h urine collection was assessed by measurement of urine creatinine (Jaffe reaction, Roche Diagnostics, Germany) and all patients had urine creatinine

concentrations within the normal reference range (9.0-18.0 μmol/24 h, in-house reference range).

Serum samples (S-monovette, Sarstedt, Germany) were collected from patients at the same visits as urine samples. Samples were centrifuged (10 min at 3000 rpm) and stored at -20 °C until analysis. All serum samples were collected following an overnight fast (at least 8 h). Patients' dietary intake of protein was managed through a 7 day food diary by a combination of lower protein in diet and phenylalanine/tyrosine free meal exchanges.

6.3.2 Methods

6.3.2.1 Beck's depression inventory-II

BDI-II (Beck *et al.,* 1961; Beck *et al.,* 1996) is a self-scored questionnaire that includes 21 items; each has 4 alternative statements ranked in order of severity from 0-3. The questionnaire evaluates a number of emotions including: mood, pessimism, sense of failure, self-dissatisfaction, guilt, punishment, self-dislike, self-accusation, suicidal ideas, crying, irritability, social withdrawal, insomnia, and loss of appetite. Conventional cut-offs are 0–13 for minimal depression, 14-19 for mild depression, 20-28 for moderate depression and 29-63 for severe depression, with a maximum score of 63. In this study all BDI-II assessments were carried out by the same practitioner.

6.3.2.2 Urine metadrenalines

The concentrations of urinary NMA, metadrenaline (MA) and 3-MT were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described by Davison *et al.* (2018b). 20 μL of sample extract was injected onto a C_{18} phenyl-hexyl column (4.6 x 100 mm, 2.6 μm, Phenomenex, Cheshire) using a Waters Acquity UPLC separations module coupled to a Xevo TQS tandem mass spectrometer. Initial conditions of 90:10 water:methanol with 0.1 % formic acid (v/v) increased linearly to 40:60 over 2 min, returning to starting conditions by 4 min. Commercial calibration standards (Chromsystems, Munich) and quality control materials (Recipe ClinChek, Germany) were used. Calibrator concentrations were 0-46.8 μmol/L for NMA; 0-24.8 μmol/L for MA and 0-27.7 μmol/L for 3-MT.

Interassay coefficient of variation for NMA, MA and 3-MT were 4.6, 4.7 and 7.2 % at 1.7, 0.8 and 1.6 μmol/L, respectively.

6.3.2.3 Urine 5-HIAA

The concentration of urinary 5-HIAA was also determined by LC-MS/MS as previously reported by Davison *et al.* (2018b). 20 μL of diluted urine sample was injected onto an Atlantis dC_{18} column (3.0 x 100 mm, 2.6 μm, Waters, Milford) using a Waters Alliance 2795 separations module coupled to a Waters Quattro Premier XE tandem mass spectrometer. Initial conditions of 95:5 water:methanol with 0.1 % formic acid (v/v) and 2 mmol/L ammonium acetate, increased linearly to 5:95 over 2 min, returning to starting conditions by 4 min. Commercial calibration standards (3.6-356 μmol/L, Chromsystems, Germany) and quality control materials (Recipe ClinChek, Germany) were used. Interassay coefficient of variation was 7.2 % for 5-HIAA at 26.2 μmol/L.

6.3.2.4 Serum amino acids

Serum amino acid concentrations were determined as previously reported by Davison *et al.* (2018a). In brief, analyses were performed using the Biochrom 30 high performance liquid chromatography cation exchange system with ninhydrin detection (Bichrom, Cambridge, UK).

Calibration was performed using a series of aqueous standards purchased from Sigma (Dorset, UK) [amino acids basic standard (product code: A6282); amino acids acidic/neutral standard (product code: A6407)].

100 μL of calibrator/serum/internal quality control material was mixed with 100 μL of a solution containing sulfosalicylic acid (183 mmol/L) to precipitate proteins and internal standards (S-2-amino-ethyl-L-cystine hydrochloride and norleucine both at 100 μmol/L). Samples were then refrigerated at 4 °C for 1 h, centrifuged at 13 000rpm for 10 min and 60 μL of supernatant injected onto the analytical column for analysis. Commercial internal quality control material was used (Recipe, Clinchek, Germany). Interassay coefficient of variation for tyrosine, phenylalanine and tryptophan were 2.5, 1.8 and 1.0 % at 68.3, 78.1 and 53.5 μmol/L, respectively.

6.3.2.5 Data analysis and calculations

Amino acid concentrations were calculated off-line using EZCHrom Elite software (Build 3.2.1 Agilent, USA). NMA, MA, 3-MT and 5-HIAA concentrations were calculated using MassLynx software (Version 4.1, Waters, USA).

All statistical analyses were performed using Graphpad Instat (Version 3.10, 2009, CA, USA) and Analyse-It for Microsoft Excel (Version 2.20 Analyse-it Software, Ltd, Leeds, UK).

Kolmogorov-Smirnov testing was performed to assess if data were normally distributed. Spearmans rank correlation was used to assess if data were correlated. Wilcoxon matched-pairs signed-ranks test (2-tailed) was used to assess significant differences pre- and post-treatment with nitisinone in patients that were followed up. Mann Whitney unpaired test (2 tailed) was used to assess un-paired data. A p value <0.05 was deemed significant.

6.4 Results

6.4.1 BDI-II

Sixty-three patients completed BDI-II questionnaires at baseline pre-nitisinone treatment and 39 patients at 12 months following 2 mg daily of nitisinone treatment. Thirty-two of these patients also completed questionnaires at 24 months following 2 mg daily of nitisinone treatment.

Mean (± standard deviation) BDI-II scores at baseline (n=63), 12 (n=39) and 24 (n=32) months were 10.1(9.6); 9.8 (10.0) and 10.5 (9.9). Mean scores were not significantly different between visits ($p>0.05$) (Figure 6.1).

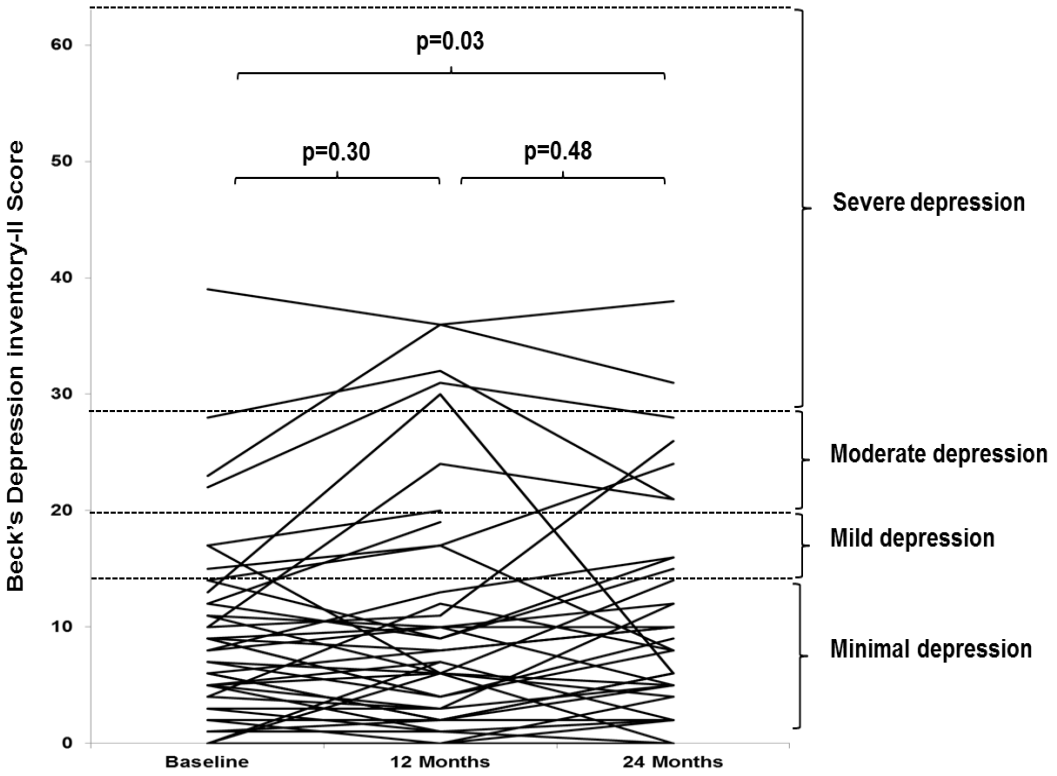

Figure 6.1. Beck's depression inventory-II data box plot at baseline (pre-nitisinone, n=39), and at 12 (n=39) and 24 months (n=32) (2 mg nitisinone daily). BDI-II scoring: minimal depression (0-13 points); mild depression (14-19 points); moderate depression (20-28 points); severe depression (29-63 points). Significance testing compared BDI-II scores at each visit. p value <0.05 was deemed significant. BDI-II scores were highly correlated at baseline and 12 months (rs=0.80, p<0.0001), baseline and 24 months (rs=0.99, p<0.0001), and 12 and 24 months (rs=0.74, p<0.0001). This supports that BDI-II scores did not change significantly in the majority of patients over the 24 month period studied.

A number of patients were followed up and so paired data were also analysed. Paired BDI-II scores were available in 39 patients at baseline and 12 months, scores were not significantly different (p=0.30). Thirty-two of the 39 patients also had BDI-II scores at 24 months (mean (±standard deviation) 10.5 (9.9) vs 8.6 (7.8), which were significantly higher compared to baseline (p=0.03). There was no significant difference between BDI-II scores at 12

and 24 months (p=0.48). BDI-II scores were not significantly different between male and female patients pre- and post nitisinone treatment at baseline (n=24 and n=15, p=0.56), 12 months (n=24 and n=15, p=0.82) and 24 months (n=18 and n=14, p>0.99).

At baseline 74.6 % (47/63) of patients had minimal depression. A similar proportion of patients that commenced nitisinone had minimal depression at 12 (30/39) and 24 months (23/32). At baseline the percentage of mild, moderate and severe depression was 11.1; 9.5 and 4.8 %, respectively. At 12 months a greater proportion of patients were classified as having severe depression (10.8 %, 4/39). At 24 months the proportion of patients with severe depression reduced to 6.3 % (2/32).

Table 6.1 shows the changes that were observed in the BDI-II classification of patients over the 24 month period studied. Six patients moved to a more severe category, and 2 patients moved to a less severe BDI-II category at 12 months. The biggest change in category was observed in patients moving from the moderate to severe category (2/7 patients). At 24 months 5 patients moved to a more severe BDI-II category and 3 to a less severe BDI-II category. The biggest change in category was in patients moving from the minimal to mild category (3/8).

BDI-II score category change	**Baseline to 12 months**	**12 to 24 months**
Minimal to mild	1	3
Minimal to moderate	1	1
Minimal to severe	1	0
Mild to moderate	1	1
Moderate to severe	2	0
Mild to minimal	2	1
Severe to moderate	0	1
Severe to minimal	0	1

Table 6.1. Beck's depression inventory-II category changes observed between baseline (pre-nitisinone), 12 and 24 months (2 mg nitisinone daily). BDI-II scoring: minimal depression (0-13 points); mild depression (14-

19 points); moderate depression (20-28 points); severe depression (29-63 points). BDI score category change in *italics* indicates improvement in category.

All patients had their medications reviewed at each visit. At baseline, 6 of the 63 patients were on anti-depressants/mood stabilising agents. Agents included: citalopram, venlafaxine, olanzapine and duloxetine. Three of these patients had BDI-II scores recorded at 12 and 24 months. Two of the 3 patients moved from the minimal to the moderate depression category.

Five patients were excluded from the 12 and 24 BDI-II data analysis as they were not residents of England or Scotland, which meant they did not receive nitisinone treatment. They still had BDI-II assessments at 12 and 24 months, so effectively acted as a control group. One of these patients showed deterioration in BDI-II category at 24 months from minimal to mild.

In addition review of medications revealed that 35 of 63 patients (56 %) were on at least 1 pain relief agent (15 patients on 1 agent; 10 patients on 2 agents; 8 patients on 3 agents and 2 patients on 4 agents). Oral medications included: tramadol, co-codamol, morphine sulphate, dihydrocodeine, aspirin, paracetamol, naproxen, co-dydramol, diclofenac, amitriptyline, pregabalin, gabapentin, and buprenorphine. Fentanyl, morphine, lidocaine and buprenorphine patches were also recorded. Patients that were followed up remained on the agents documented at baseline.

6.4.2 Urine monoamine metabolites

Urinary monoamine metabolites were measured in a sub-group of patients that had BDI-II testing carried out. This was because urine samples were not available in all patients. Urinary NMA, MA and 3-MT (Figure 6.2(A-C)) were measured at baseline (n=33), 3 months (n=29), 12 months (n=31) and 24 months (n=32). Urinary 5-HIAA (Figure 6.2(D)) was measured at the same time points but only in 21 patients.

MA concentrations were significantly lower at 3 months when compared to baseline concentrations (p=0.04). At the 3 month time point MA concentrations were all within the normal reference range and there was less of a spread of concentrations. At baseline, 12 and 24 months not all MA concentrations were within the normal reference range (Figure 6.2(A)). Interestingly the same pattern was observed in NMA concentrations; however no significant difference were observed between visits (Figure 6.2(B)).

3-MT results were significantly higher at 12 and 24 months following 2 mg daily of nitisinone treatment when compared to baseline and 3 month concentrations (2 mg nitisinone every other day) (p<0.0001) (Figure 6.2(C)). At baseline and 3 months 3-MT concentrations were within the normal reference range in the majority of patients; in contrast at 12 and 24 months 3-MT concentrations were outside the normal reference range in the majority of patients.

5-HIAA concentrations were significantly lower at 12 months when compared to baseline (p=0.03). Only 1 patient had 5-HIAA concentrations outside the normal reference range (in-house reference range <50 μmol/24 h), and this was at 3 months (Figure 6.2(D)).

As monoamine metabolites are related to catecholamine and serotonin neurotransmitters they were correlated with BDI-II scores. At baseline, 12 and 24 months BDI-II scores were not correlated with NMA, MA, 3-MT, or 5-HIAA (Table 6.2).

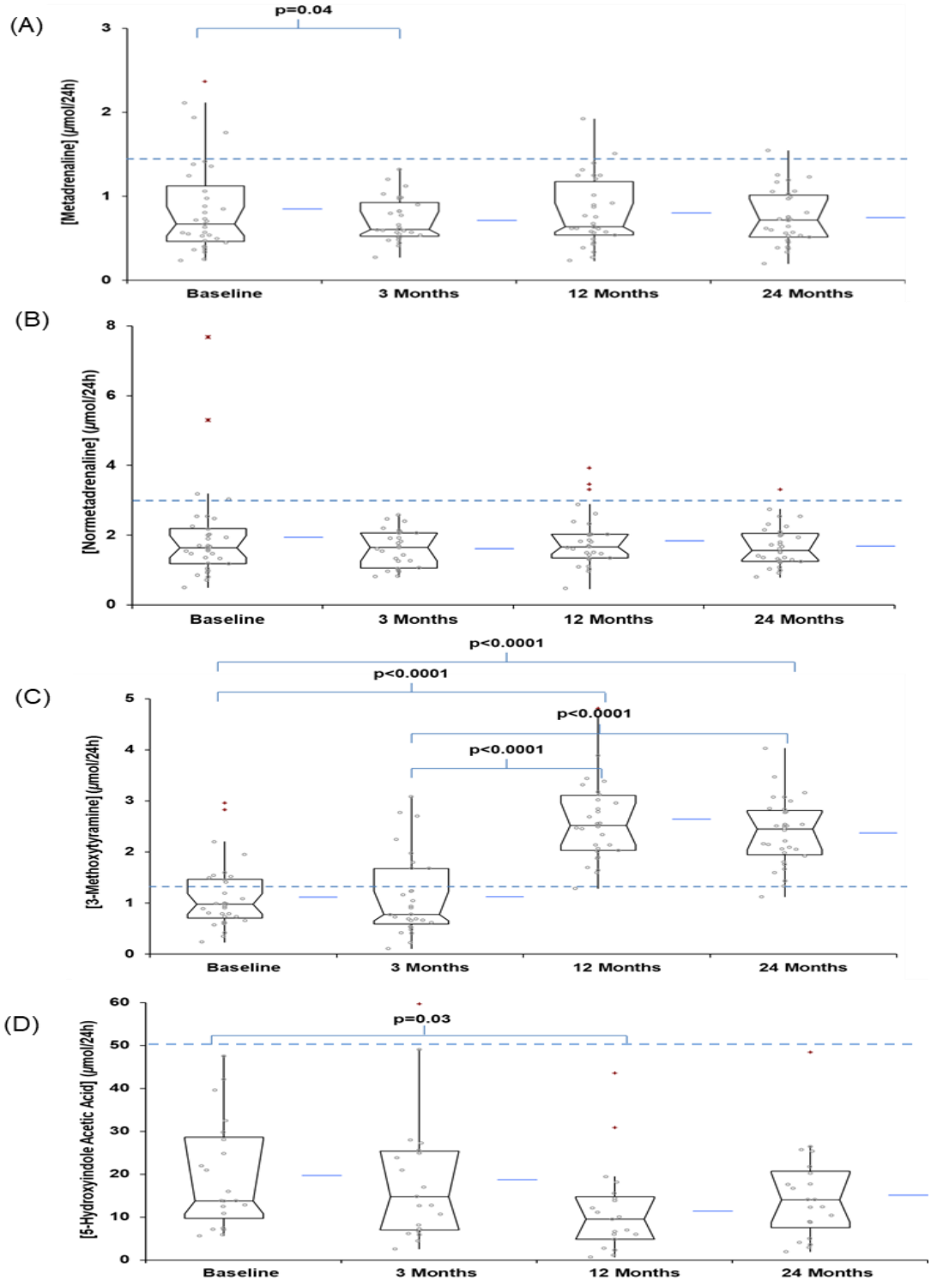

Figure 6.2. Urinary monoamine metabolite concentrations in patients with AKU. This includes samples at baseline (pre-nitisinone) (n=32 for metadrenalines, n=21 for 5-HIAA) and after treatment at 3 (n=29 for metadrenalines, n=21 for 5-HIAA), 12 (n=31 for metadrenalines, n=21 for 5-HIAA) and 24 (n=32 for metadrenalines, n=21 for 5-HIAA) months (2 mg

nitisinone daily). (A) MA; (B) NMA; (C) 3-MT and (D) 5-HIAA. --- = indicates upper limit of normal urine reference range for NMA <3.0 μmol/24 h; MA <1.3 μmol/24 h; 3-MT <1.4 μmol/24 h; 5-HIAA <50 μmol/24 h - = 95 % confidence notched outlier boxplot; + = outlier; o=individual patient concentrations. Significance testing compared monoamine concentrations before and after treatment at each visit. p value <0.05 was deemed significant

6.4.3 Serum amino acids

Serum phenylalanine, tyrosine and tryptophan were measured at baseline, 3, 12 and 24 months. Herein the concentrations of these aromatic amino acids are only included where patients also had BDI-II assessments at baseline and at follow up. This was to assess if changes in amino acid concentration were observed following nitisinone treatment.

Serum tyrosine (Figure 6.3 (A)) was within the normal reference range (29-92 μmol/L) in all patients (n=30) before nitisinone treatment. Tyrosine significantly increased (p<0.0001) following treatment with nitisinone at 3 months (n=28), 12 months (n=27) and 24 months (n=28). Serum concentrations increased 6-7 times the upper reference range on 2 mg every other day and daily. There were no significant differences in tyrosine concentrations comparing 3 months with 12 and 24 months.

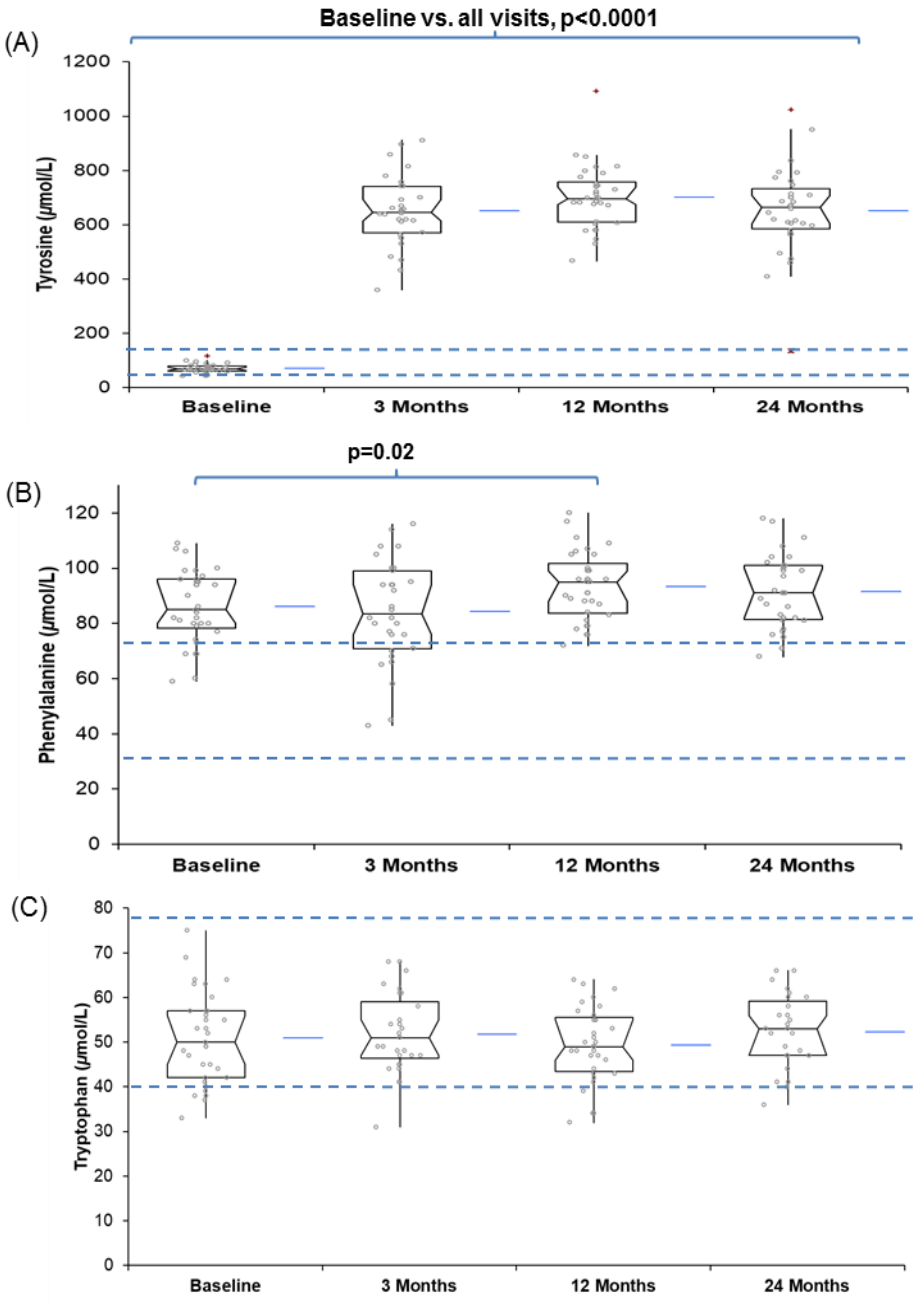

Figure 6.3. Serum amino acid concentrations in patients with AKU. This includes samples at baseline (pre-nitisinone) (n=30) and after treatment at 3 (n=28), 12 (n=27) and 24 (n=28) months (2 mg nitisinone daily). (A) Tyrosine; (B) Phenylalanine and (C) Tryptophan. --- = indicates the normal serum reference range for tyrosine 29-92 µmol/L; phenylalanine 30-76 µmol/L and tryptophan 40-79 µmol/L. Significance testing compared serum aromatic amino acid concentrations before and after treatment at each visit. p value <0.05 was deemed significant.

Phenylalanine concentrations showed a slight increasing trend (mean baseline phenylalanine=86.1 μmol/L (n=30) and mean 24 month (n=28) phenylalanine=91.7 μmol/L, (Figure 6.3(B)). Significant difference were observed between baseline and 12 months (n=27) (p=0.02). Interestingly phenylalanine concentrations were outside the upper end of the normal reference range (30-76 μmol/L) in >73 % of patients across all visits.

In contrast serum tryptophan concentrations did not significantly change from baseline (n=30) following nitisinone therapy at 3 (n=28), 12 (n=27) or 24 months (n=28), and concentrations remained within the normal reference range (40-79 μmol/L) in the >80 % of patients across all visits (Figure 6.3(C)).

The aromatic amino acids tyrosine, tryptophan and phenylalanine were correlated with BDI-II scores (Table 6.2) as all 3 amino acids play a key role in neurotransmitter biosynthesis. A weak positive correlation was observed between baseline BDI-II scores and tyrosine only (rs=0.41, p=0.02).

Variables	Baseline		12 months		24 months	
	Correlation coefficient (rs) (95 % CI)	p value	Correlation coefficient (rs) (95 % CI)	p value	Correlation coefficient (rs) (95 % CI)	p value
BDI-II vs. NMA (n=25)*	0.39 (0 to 0.68)	0.05	-0.18 (-0.55 to 0.25)	0.41	-0.05 (-0.44 to 0.36)	0.82
BDI-II vs. MA (n=25)*	0.26 (-0.15 to -0.59)	0.21	-0.23(-0.59 to 0.20)	0.28	0.02 (-0.38 to 0.42)	0.86
BDI-II vs. 3-MT (n=25)*	0.25(-0.16 to -0.59)	0.22	-0.02 (-0.43 to 0.39)	0.91	0.04 (-0.37 to 0.32)	0.90
BDI-II vs. 5-HIAA (n=20)**	0.05 (-0.41 to 0.50)	0.81	-0.20 (-0.59 to 0.27)	0.39	-0.12 (-0.60 to 0.41)	0.65
BDI-II vs. Phenylalanine (n=30)***	0.23 (-0.14 to 0.55)	0.21	0.41 (0.03 to 0.68)	0.06	0.12 (-0.27 to 0.46)	0.55
BDI-II vs. Tyrosine (n=30)***	0.41 (0.06 to 0.67)	0.02#	0.03 (-0.35 to 0.40)	0.88	0.13 (-0.25 to 0.48)	0.49
BDI-II vs. Tryptophan (n=30)***	-0.08 (-0.43 to 0.29)	0.68	0.06 (-0.33 to 0.44)	0.76	-0.25 (-0.59 to 0.17)	0.24

Table 6.2. Correlations between Beck's depression inventory-II scores and (1) Monoamine neurotransmitter metabolites and (2) Aromatic amino acids at baseline (pre-nitisinone) and at 12 and 24 months (2 mg nitisinone daily). *=24 patients at 24 months with BDI-II and metadrenaline results; **=15 patients at 24 months with BDI-II and 5-HIAA results. ***=27 patients at 12 months and 28 patients at 24 months; # = significance, p value <0.05 was deemed significant. CI – confidence interval; 5-HIAA – 5-hydroxyindole acetic acid; 3-MT – 3-methoxytyramine; MA – metadrenaline; NMA - normetadrenaline.

6.5 Discussion

Currently nitisinone is being evaluated off licence at the NAC in Liverpool for treatment of AKU. Concerns exist over its use, in particular there is a focus on whether the hypertyrosinaemia may alter neurotransmitter metabolism and thus lead to altered mood or depression. Herein we report BDI-II scores as a self-reporting measure of depression before and after treatment with nitisinone, along with biochemical markers associated with depression.

6.5.1 Beck's depression inventory-II

Mean BDI-II scores were not significantly different pre- and post-nitisinone treatment at 12 and 24 months, and the majority of patients were in the minimal depression category. This is the least severe category for depressive symptom scores.

Interestingly BDI-II scores were shown to be significantly higher when paired data were compared at baseline and 24 months (n=32, p=0.03); mean scores were 8.6 and 10.5, respectively. It is important to note that despite this the majority of patients still fell into the least severe category for depression. This significant increase was not observed when comparing baseline and 12 month results. Moreover there were no significant differences observed with respect to gender although mean BDI-II scores were higher in men. The increase in BDI-II scores observed may reflect natural variation in the scores generated in a self-reporting questionnaire, it is important to recognise that while BDI-II assessments are widely accepted in medical practice due to the ease of implementation and cost effectiveness they are challenging to use in medically ill patients as the presence of somatic symptoms in physical disease can mislead their score interpretation (Wang *et al.,* 2013). Patients included in this study have chronic pain, as confirmed by 56 % of patients being on 1 or more pain relieving agents. It is highly likely this will have influenced BDI-II scores, but as all patients remained on medications for the duration of the study, data are not biased.

A small proportion of patients changed BDI-II category; in the first 12 months 6 of 8 patients moved into a more severe category and 2 of 8 to a less severe category. The greatest changes were in patients moving from the moderate to severe (n=2), and mild to minimal (n=2) categories. These changes are probably not attributable to the effect of nitisinone directly, but are more likely a reflection of the variation seen in a self-reporting questionnaire and the impact of living with a chronic painful musculoskeletal disease and its associated comorbidities. At 24 months the same number of patients moved category, 5 of 8 moving into a more severe category and 3 of 8 improving. The biggest changes were observed in the movement from the minimal to mild category (n=3). Interestingly 2 of 3 patients that showed improvements in category moved from severe to moderate and minimal categories, respectively.

In all of the BDI-II questionnaires carried out 9.5, 7.6 and 9.3 % of patients were taking antidepressants at baseline, 12 and 24 months. The prevalence of patients taking anti-depressants in this study is very similar to reported national patterns (Olfson *et al.,* 2009). It is surprising that such a small proportion of patients were taking anti-depressants given the chronic pain and debilitation symptoms these patients have. This is likely to be a reflection that the majority of patients were not depressed and thus medication was not required, and a large proportion of patients were on pain relief medication. This is in contrast to a study that examined depression in patients with a similarly debilitating disease, osteoarthritis where in patients over the age of 65 years (n=1425), 23.1 % were taking anti-depressant medication (Gleicher *et al.,* 2011).

Patients that were taking anti-depressant medication were not excluded from the data as they represent the typical population and were believed not to skew the data. Two patients taking antidepressant medications changed category, but this is not believed to be due to the effect of nitisinone.

In this longitudinal survey 5 patients were excluded from the data analysis at 12 and 24 months as they did not receive nitisinone. By definition these

patients acted as a ‘control group’, mean BDI scores were 10.6 and 10.8 at 12 and 24 months, respectively. This is very similar to those patients receiving nitisinone (9.8 and 10.5 at 12 and 24 months, respectively) and thus while this is a small group it supports that nitisinone is not having a detrimental impact on mood.

6.5.2 Urinary monoamine metabolites and serum amino acids

In patients where urine and serum samples were available urinary monoamine metabolites and serum aromatic amino acids were measured to assess if treatment with nitisinone affected their concentrations. This is of particular importance because the aromatic amino acids are precursors to the catecholamine and serotonergic neurotransmitters, and urinary monoamine metabolites are a biochemical reflection of peripheral neurotransmitter metabolism; both have previously been shown to be altered in patients with depression (Roy *et al.,* 1986a; Roy *et al.,* 1986b; Grossman *et al.,* 1999; Hughes *et al.,* 2004).

In keeping with previous (Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Davison *et al.,* 2018b) serum tyrosine increased significantly to outside of the normal reference range at all visits following 2 mg nitisinone treatment. This marked increase resulted from the action of nitisinone, which is a reversible competitive inhibitor of the HPPD enzyme.

The increase in tyrosine was accompanied by a small, but significant increase in phenylalanine concentrations when baseline was compared to 12 months. Of note is that phenylalanine concentrations were outside the normal reference range (30-76 *μ*mol/L, in-house reference range) independently of treatment with nitisinone. This has been previously reported by Davison *et al.* (2018b). It has been postulated that while these changes were significant, they may reflect normal intra-individual variation in amino acid concentration. It is also possible that changes result from reduced phenylalanine hydroxylase activity. Reduced enzyme activity has been reported in individuals with increased inflammation (Wannemacher *et al.,* 1976). As AKU

is a chronic disease process it is possible that inflammatory processes are altered, however this was not assessed.

Changes in serum amino acids were accompanied by a significant increase in the urinary DP metabolite 3-MT at 12 and 24 months, where the majority of patients had concentrations outside the normal reference range (<1.4 *μ*mol/24 h). Patients with 3-MT outside the normal reference range were also observed at baseline and 3 months. It is thought that this may reflect dietary intake of foods containing biogenic amines (De Jong *et al.,* 2009). No patients with increased 3-MT at these time points were on any medications (including anti-depressants) known to increase 3-MT concentrations. This is consistent with previous findings (Davison *et al.,* 2018b) in patients taking a 2 mg daily dose of nitisinone.

The marked increase in 3-MT excretion indicates that nitisinone therapy alters the metabolism of catecholamines, specifically DP. As previously highlighted (Davison *et al.,* 2018b) the metabolism of catecholamine neurotransmitters is complex and they have multiple origins including the sympathetic nerves (involved peripheral metabolism), adrenal medulla, brain (involved in central nervous system metabolism) and mesenteric organs (Eisenhofer *et al.,* 2004). It has been demonstrated that the concentration of monoamine neurotransmitters in urine are dependent on plasma concentrations and uptake via organic cation transporters in the kidney (Eisenhofer *et al.,* 1996; Graefe *et al.,* 1997). It is also important to consider the degree to which renal neurotransmitter synthesis can contribute to the urinary concentrations.

Davison *et al.* (2018b) suggested the increase in 3-MT is highly likely to reflect renal synthesis as serum tyrosine concentrations increased significantly post nitisinone therapy. It is thought that the increased tyrosine load delivered to the kidney may provide a substrate for the synthesis of dihydroxyphenylalanine, and subsequent decarboxylation to DP may lead to the increase in 3-MT observed. However this rationale does not explain why changes in 3-MT were not observed at 3 months after nitisinone therapy

every other day, despite the large increase in serum tyrosine concentration. The explanation for this is unknown and requires further investigation.

This supposition is supported by BDI-II scores not changing drastically and the majority of patients did not move to a more severe category of depression. In contrast Roy *et al.* (1986b) showed that the urinary metabolite of DP, dihydroxyphenylacetic acid was significantly lower in patients with depression, and suggested that this finding shows dysregulation of the sympathetic nervous system in depression.

Urinary MA was significantly lower between baseline and 3 months following nitisinone treatment, but not at 12 and 24 months. This is in contrast to previous (Davison *et al.,* 2018b) where no significant changes were observed following nitisinone treatment. This study however showed that urinary MA concentrations were less variable following nitisinone treatment (Davison *et al.,* 2018b). The lack of significantly lower MA concentrations across all visits suggests this finding is not due to nitisinone therapy and may reflect variation in dietary intake (de Jong *et al.,* 2009).

While there were no significant differences observed in urinary NMA concentrations following treatment; at 3 months urinary excretion also showed less variation compared to all other visits. As this is not a consistent finding at other visits it is of unknown significance and requires further investigation. Previously Davison *et al.* (2018b) showed that NMA decreased significantly following a daily dose of nitisinone at 1-8 mg. The reason for not seeing this difference in the current study is unknown; the findings presented herein are however based on a larger cohort of patients that were followed up over a 24 month period and likely to be more representative of the effect of nitisinone on urinary NMA excretion.

Previous studies (Roy *et al.,* 1986a; Roy *et al.,* 1986b; Grossman *et al.,* 1999; Hughes *et al.,* 2004) have demonstrated that urinary concentrations of NA, MA and adrenaline were significantly higher in subjects with depression compared to controls and concluded that this reflected dysregulation of the

sympathetic nervous system in depression. In contrast, the majority of patients in our study were in the least severe category of depression, which supports the biochemical findings presented.

Herein the mean urinary excretion of 5-HIAA decreased following treatment with nitisinone at all visits (only significantly lower at 12 months), suggesting that peripheral serotonin metabolism is altered. This however was not reflected in changes in BDI-II scores. In contrast Davison *et al.* (2018b) only showed a significant decrease in 5-HIAA excretion following a daily 8 mg dose of nitisinone. This difference may be the result of the current study being over a 24 month period and not 8 weeks.

Thimm *et al.* (2011) has reported alterations in serotonin metabolism in patients with HT1 treated with nitisinone. This study showed a decrease in cerebrospinal fluid (CSF) 5-HIAA concentrations following nitisinone therapy. It is proposed that the decrease in CSF 5-HIAA occurred as increased tyrosine concentrations compete with tryptophan (serotonin precursor) via a neutral amino acid transporter across the blood-brain barrier, thus reducing tryptophan availability for intracerebral serotonin synthesis (Pratt *et al.,* 1982).

In addition it has been hypothesised that elevated tyrosine may inhibit tryptophan hydroxylase activity, the rate limiting step for serotonin metabolism (Thimm *et al.,* 2011). Although this study was small and analysis was performed in CSF it has been shown that urinary analysis of serotonin and 5-HIAA reflect parallel changes in immunoreactivity in the dorsal raphe nucleus, demonstrating a positive correlation between CNS serotonergic activity and urinary serotonin concentrations (Lynn-Bullock *et al.,* 2004).

6.5.3 Correlation between BDI-II scores and biochemical measurements

A weak positive correlation was observed between BDI-II scores and serum tyrosine before nitisinone treatment. This is not unexpected as tyrosine is the precursor to catecholamine neurotransmitters. Following nitisinone treatment

and consequent hypertyrosinaemia, this correlation disappeared. This is likely to reflect the large rapid change in tyrosine with very little change in BDI-II scores. This supports the postulate that hypertyrosinaemia does not result in worsening mood or depression in this cohort. The significant increase in the ratio of tyrosine to large neutral amino acids (*i.e.* phenylalanine and tryptophan) has been previously reported following nitisinone treatment (Davison *et al.,* 2018b), and it is has been hypothesised that hypertyrosinaemia may reduce phenylalanine and tryptophan uptake into the brain due to competition for the LAT-1 transporter (van Ginkel *et al.,* 2016), thus altering neurotransmitter metabolism. As serum amino acids were measured in serum, speculative inferences can only be made as concentrations do not necessary reflect the concentration in CSF.

The lack of correlation between BDI-II scores and monoamine metabolites is not surprising for a number of reasons. Firstly, while urinary monoamine metabolites are produced by the sympathetic nervous system and adrenal gland, they also reflect production in the mesenteric organs and dietary intake thus reducing their specificity as a marker of increased sympathoadrenal activity. Moreover the majority of patients included herein were not clinically depressed before or after nitisinone treatment.

6.6 Conclusions

BDI-II scores were significantly higher after 24 months of nitisinone treatment in patients that were followed up. Despite this the majority of patients remained in the least severe category of depression suggesting that nitisinone treatment does not substantially worsen mood. Independent of this there were significant effects on serum tyrosine concentrations and monoamine neurotransmitter metabolism in patients following treatment with nitisinone.

6.7 Declaration and acknowledgements

Preparation, analysis and data processing associated with urine samples for measurement of metadrenalines and 5-HIAA from patients attending the NAC was performed by Andrew Davison.

Preparation and analysis of serum samples for amino acids was performed by Andrew Davison and Elizabeth Smith (Department of Clinical Biochemistry, Alder Hey Children's Hospital, Liverpool, UK). Serum amino acid and BDI-II data analysis performed by Andrew Davison.

BDI-II data were collected by Gin Hughes and Joanne Harold (Department of Psychology, University of Liverpool, UK).

Jean Devine (Department of Clinical Biochemistry, Liverpool Clinical Laboratories, Royal Liverpool Hospital, Liverpool, UK) processed all serum and urine samples from patients attending the NAC.

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Chapter 7

Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging

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7.1 Abstract

Background: Nitisinone induced hypertyrosinaemia is a concern in patients with Alkaptonuria (AKU). It has been suggested that this may alter neurotransmitter metabolism, specifically dopamine and serotonin. Herein mass spectrometry imaging (MSI) is used for the direct measurement of 2,4-diphenyl-pyranylium tetrafluoroborate (DPP-TFB) derivatives of monoamine neurotransmitters in brain tissue from a murine model of AKU following treatment with nitisinone.

Materials and methods: Metabolite changes were assessed using MSI on DPP-TFB derivatised fresh frozen tissue sections directing analysis towards primary amine neurotransmitters. Matched tail bleed plasma samples were analysed using LC-MS/MS. Eighteen BALB/c mice were included in this study: *HGD*$^{-/-}$ (n=6, treated with nitisinone – 4 mg/L, in drinking water); *HGD*$^{-/-}$ (n=6, no treatment) and *HGD*$^{+/-}$ (n=6, no treatment).

Results: Ion intensity and distribution of DPP-TFB derivatives in brain tissue for dopamine, 3-methoxytyramine, noradrenaline, tryptophan, serotonin, and glutamate were not significantly different following treatment with nitisinone in *HGD* $^{-/-}$ mice, and no significant differences were observed between *HGD*$^{-/-}$ and *HGD*$^{+/-}$ mice that received no treatment. Tyrosine (10-fold in both comparisons, p=0.003; [BALB/c *HGD*$^{-/-}$ (n=6) and BALB/c *HGD*$^{+/-}$ (n=6) (no treatment) *vs*. BALB/c *HGD*$^{-/-}$ (n=6, treated)] and tyramine (25-fold, p=0.02; 32-fold, p=0.02) increased significantly following treatment with nitisinone. Plasma tyrosine and homogentisic acid increased (9-fold, p<0.0001) and decreased (9-fold, p=0.004), respectively in *HGD*$^{-/-}$ mice treated with nitisinone.

Conclusions: Monoamine neurotransmitters in brain tissue from a murine model of AKU did not change following treatment with nitisinone. These findings have significant implications for patients with AKU as they suggest monoamine neurotransmitters are not altered following treatment with nitisinone.

7.2 Introduction

Alkaptonuria (AKU, OMIM 203500) is a rare bi-allelic autosomal recessive disorder of the tyrosine metabolic pathway, occurring 1 in 100,000-250,000 of the general population (Phornphutkul *et al.,* 2002) and arises from a congenital deficiency in the enzyme homogentisate 1,2-dioxygenase (HGD, E.C.1.12.11.5) (Figure 7.1). The biochemical hallmark of AKU is that circulating concentrations of homogentisic acid (HGA) markedly increase, and it is this that is thought to be responsible for many of the complications observed (for a detailed review see Ranganath *et al.,* 2013). Treatment for this condition is largely based on supportive and palliative measures that include pain relief and anti-inflammatory medications, dietary protein restriction and joint replacement (Ranganath *et al.,* 2013). Currently the drug nitisinone, a competitive inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C.1.13.11.27) (Figure 7.1), is being evaluated as a potential treatment (Ranganath *et al.,* 2016; Milan *et al.,* 2017; SONIA-2 – ClinicalTrials.gov Identifier: NCT01916382). This drug acts to move the metabolic block in the tyrosine metabolic pathway resulting in a marked reduction in the circulating concentration of HGA and a consequential significant increase in tyrosine, creating a so called 'pseudo type-3 Tyrosinaemia' picture (Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Olsson *et al.,* 2015; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Davison *et al.,* 2018b; Davison *et al.,* 2018c). Hypertyrosinaemia has also been well documented in patients with hereditary tyrosinaemia type 1 (HT1, OMIM 276700) that are treated with nitisinone (Lindstedt *et al.,* 1992; van Ginkel *et al.,* 2017; Zeybek *et al.,* 2017).

The metabolic fate of these supraphysiological tyrosine concentrations is unknown. It has been proposed in HT1 that hypertyrosinaemia may contribute to the neurodevelopmental delay that is frequently observed in children (McKiernan *et al.,* 2015). Several mechanisms have been proposed for this, including increased transport of tyrosine into the brain; decreased transport of other neutral amino acids into the brain (specifically tryptophan); increased central nervous system (CNS) dopamine (DP); decreased CNS

serotonin, oxidative damage from δ-aminolevulinic acid and succinylacetone or modification of neuronal proteins (Thimm *et al.,* 2011; Hillgartner *et al*., 2016). It has also been suggested that altered serotonin metabolism may be due to direct inhibition of tryptophan hydroxylase (TPH, EC.1.14.16.4) activity by tyrosine, which leads to a reduced biosynthesis of serotonin (Thimm *et al.,* 2011).

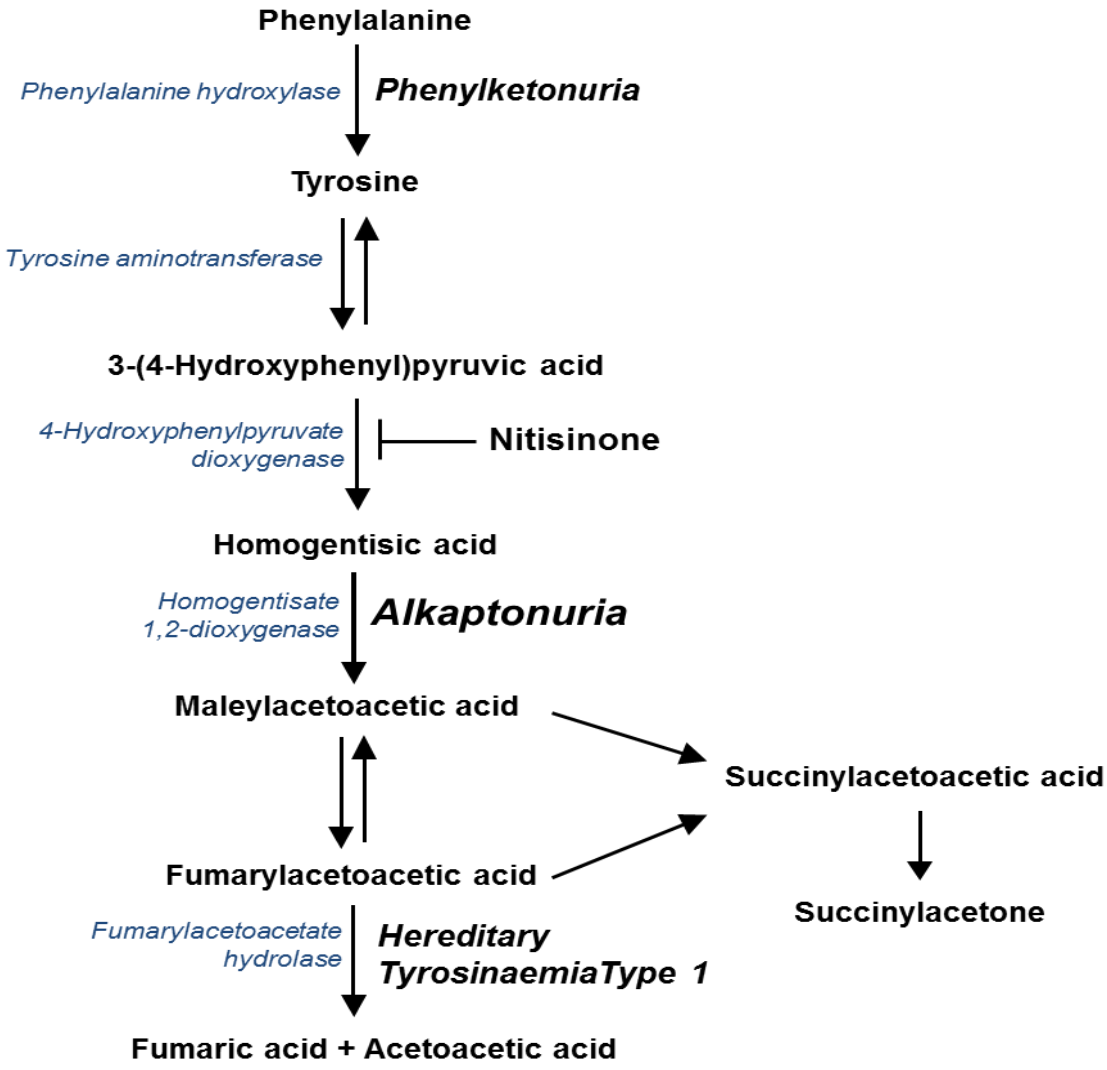


Figure 7.1. Tyrosine metabolic pathway. Highlighted are the enzyme defects observed in Phenylketonuria (phenylalanine hydroxylase, PAH E.C.1.14.16.1), AKU (homogentisate 1,2-dioxygenase, HGD E.C.1.13.11.5) and HT1 (fumarylacetoacetate hydrolase, FAH E.C.3.7.1.2), and the site of action of nitisinone inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C.1.13.11.27). Adapted from Davison *et al.* (2018b).

In AKU there is uncertainty about whether the hypertyrosinaemia may alter neurotransmitter metabolism and specifically whether this may lead to depression or altered cognition through the mechanisms detailed above. Recently Davison *et al.* (2018c) concluded that treatment with nitisinone is unlikely to cause depression in patients with AKU in a study that assessed urinary neurotransmitter metabolite concentrations, in a cohort of patients with AKU. This study did show an increase in urinary 3-methoxytyramine (3-MT, DP metabolite) and a decrease in urinary 5-hydroxyindole acetic acid (serotonin metabolite) following treatment. These changes did not correlate with Beck's depression inventory-II scores. Similar biochemical findings were also reported in a study that evaluated different doses of nitisinone (0-8 mg daily for 4 weeks) in patients with AKU over a 4 week period (Davison *et al.,* 2018b).

While previous studies have demonstrated that hypertyrosinaemia clearly results in altered peripheral metabolism of dopaminergic and serotoninergic neurotransmitter metabolites they are limited as they are not a direct reflection of neurotransmitter metabolism in the CNS, which is directly linked to mood and cognition. Direct measurement of neurotransmitter concentrations in cerebrospinal fluid and or in the brain are not feasible in this patient group owing to their complex musculoskeletal comorbidities.

Herein for the first time we report a direct approach to assess whether hypertyrosinaemia effects monoamine neurotransmitter metabolism. Desorption electrospray ionisation mass spectrometry imaging (DESI-MSI) was used to measure the ion intensity and distribution of 2,4-diphenyl-pyranylium tetrafluoroborate (DPP-TFB) derivatives of monoamine neurotransmitters in brain tissue from a murine model of AKU (BALB/c *HGD*$^{-/-}$) (Preston *et al.,* 2014) following treatment with nitisinone. Applying an imaging approach will enable us to identify whether any occurring changes are only appearing in certain brain sub-structures or are systemic to the whole brain. Chemical charge tagging of monoamine neurotransmitters (primary amines) with DPP-TFB was carried out as previous studies have demonstrated significant improvements in signal intensity compared to their

analysis in native brain tissue (Shariatgorji *et al.,* 2014; Esteve *et al.,* 2016; Shariatgorji *et al.,* 2016).

DESI-MSI was employed over other techniques as it allows the direct mapping of the distribution and localisation of multiple molecular species in a single experiment (Shariatgorji *et al.,* 2014; Shariatgorji *et al.,* 2016). This is in contrast to the direct measurement of individual metabolite concentrations in brain tissue homogenates, which gives no information on distribution and localisation, and more traditional indirect methods like histological, immunochemical and ligand based assays (de Jong *et al.,* 2005).

In addition plasma concentrations of HGA and tyrosine were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS), to confirm that the murine model employed (1) had the expected biochemical phenotype observed in AKU (*i.e.* elevated HGA) and (2) hypertyrosinaemia was observed following treatment with nitisinone.

7.3 Materials and methods

7.3.1 Chemicals and reagents

Triethylamine (TEA), hydroxypropylmethylcellulose, polyvinylpyrrolidone and perchloric acid were purchased from Sigma-Aldrich (Dorset, UK). Water, methanol and trifluoroacetic acid (TFA) were obtained from Merck (Hohenbrunn, Germany). DPP-TFB was obtained from American Custom Chemicals Corporation (San Diego, CA, USA).

7.3.2 Animal experiments

7.3.2.1 Protocol for treating murine AKU model with nitisinone

A murine model of AKU was used for all experiments as described previously (Preston *et al.,* 2014). Eighteen BALB/c mice (Figure 7.2) (12 $HGD^{-/-}$ and 6 $HGD^{+/-}$) were used in total. Six mice ($HGD^{-/-}$) were administered nitisinone (4 mg/L) through drinking water for one week, the remaining 12 mice (6 $HGD^{-/-}$ and 6 $HGD^{+/-}$) received no nitisinone. All animals were housed in air

conditioned rooms (with a 12 h dark/light cycle) at 20 °C and 53 % humidity, with access to food and water *ad libitum* at the University of Liverpool. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

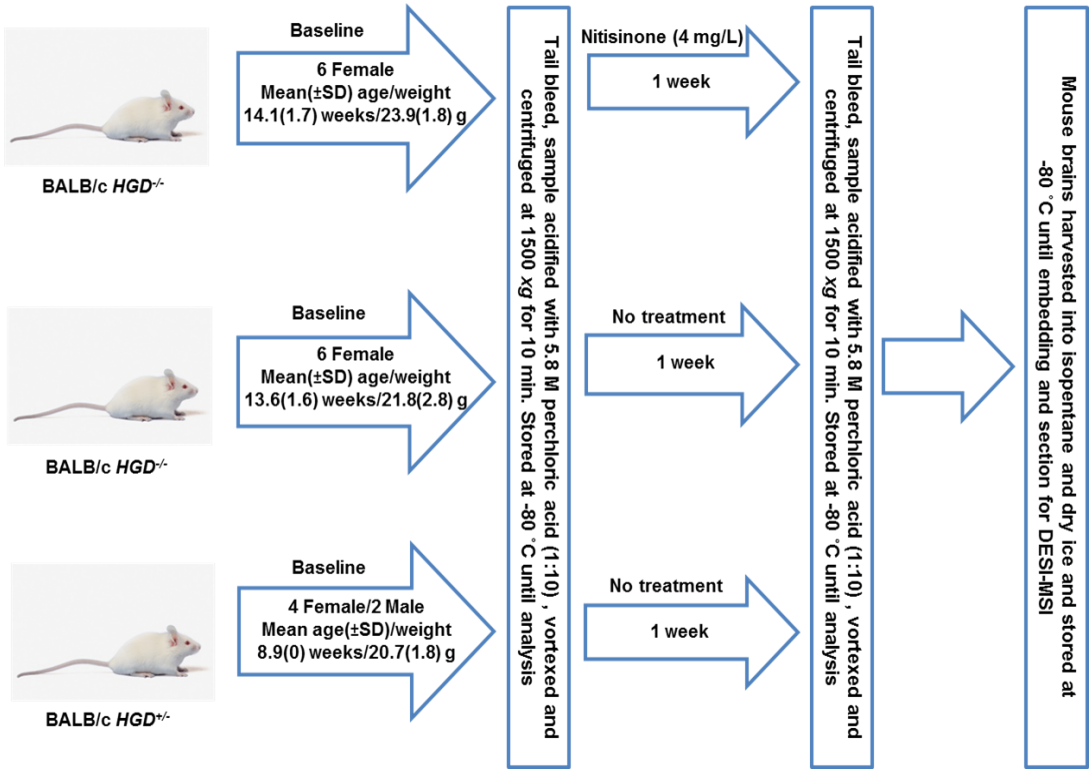


Figure 7.2. Summary of experimental workflow involving murine model of AKU. Included are mouse gender, weight and age; treatment groupings and sample collection, processing and storage. SD – standard deviation; g – grams.

7.3.2.2 Collection of tail bleed samples

Tail bleed samples were collected into Microvettes (Sarstedt, Germany) at baseline and after 1 week prior to culling. Whole blood samples were centrifuged at 1500 ×*g* for 10 min at 4 °C and the plasma deproteinised by adding 60 % 5.8 M perchloric acid (ratio 1:10, perchloric acid:plasma).

Samples were vortexed and centrifuged at 1500 ×*g* for a further 10 min. The supernatant was stored at -80 °C until analysis (Figure 7.2).

7.3.2.3 Brain harvesting

Brains were resected from each animal following cervical dislocation, and placed into a trough of dry ice cooled isopentane until any effervescence stopped. They were then removed using chilled forceps and wrapped in aluminium foil and stored at -80 °C until dissection.

7.3.2.4 Brain tissue processing and derivatisation

Frozen brain tissues were placed into plastic moulds and embedded into a matrix composed of 7.5 % (w/v) hydroxypropylmethylcellulose and 2.5 % (w/v) polyvinylpyrrolidone. Brains were divided into 3 moulds (n=6 per mould, 2 brains from each treatment group) to allow axial (n=6), sagittal (n=6) and coronal (n=6) sectioning to allow spatially resolved assessment of metabolite changes in brain substructures. This embedding approach was chosen in order to ensure that all brains are treated identically as it was previously shown that consecutive sectioning can lead to metabolite degradation (Swales *et al.,* 2018). Although it was attempted to position brains into the moulds in exactly same position and orientation, this approach might lead to slight differences in sectioning depth and position.

Embedded tissues were then cut using a cryo-microtome operated at -20 °C (Leica CM3050S, Leica Microsystems, Wetzlar, Germany) to a thickness of 10 *µ*m and thaw mounted onto superfrost microscope glass slides (Thermo Scientific, Braunschweig, Germany). These slides were subsequently dried under a gentle flow of nitrogen before being stored at −80 °C until derivatisation and MSI analysis.

Brain tissues were derivatised as previously described (Shariatgorji *et al.,* 2014; Shariatgorji *et al.,* 2016) using DPP-TFB (9.2 mg DPP-TFB was dissolved in 7.2 mL of 75 % methanol alkalinised with 3.5 *µ*L of TEA to obtain a 1.3 mg/mL derivatisation solution). This was applied using a TM sprayer (HTX Technologies, Chapel Hill, NC) at 75 °C in 30 passes using criss-cross

pattern [flow rate 80 *μ*L/min, nitrogen flow of 10 psi, nozzle speed 1100 mm/min]. Chemical charge tagging of primary amine functional groups with DPP-TFB was done to enhance signal intensity of monoamine neurotransmitters as their analysis has proven difficult due to their low-ionization efficiency, spectral interferences from tissue components, ion suppression effects and analyte insource fragmentation (Shariatgorji *et al.,* 2014; Esteve *et al.,* 2016; Shariatgorji *et al.,* 2016).

7.3.3 Analytical methods for the analysis of murine samples

7.3.3.1 Liquid chromatography tandem mass spectrometry analysis of murine plasma samples

Plasma samples from tail bleeds were analysed for tyrosine, nitisinone and HGA using a previously published method (Hughes *et al.,* 2015). In brief, samples were analysed for all 3 analytes in a single run on an Agilent 6490 triple quadrupole tandem mass spectrometer with Jet-Stream® electrospray ionization coupled with an Agilent 1290 Infinity II UHPLC pump. Separation was achieved on an Atlantis dC18 column (3.0 x 100 mm, 3 *μ*m, Waters) maintained at 35 °C. Quantification was achieved using a matrix matched 7 point calibration curve and 2 product ion transitions for each analyte of interest (HGA 167>122 and 167>108, negative polarity; tyrosine 182>136 and 182>91; nitisinone 330>218, positive polarity).The linear measuring range for tyrosine, HGA and nitisinone were 60-2000; 15-500 and 0.5-10 *μ*mol/L, respectively. 2 *μ*L of deproteinized sample was diluted 1:1000 with 200 nmol/L $^{13}C_6$-HGA, 500 nmol/L d_4-tyrosine and 2 nmol/L $^{13}C_6$-nitisinone in 0.1 % formic acid/deionized water. 10 *μ*L of diluted plasma was injected onto the column. Data were acquired using MassHunter LC/MS Data Acquisition (Build 07.00).

7.3.3.2 Mass spectrometry imaging of murine brain tissue

MSI data were acquired using an OmniSpray-2D DESI ion source (Prosolia Inc, Indianapolis, IN, USA) mounted onto a Q-Exactive Plus mass spectrometer (Thermo Scientific, Bremen, Germany). A home-built DESI sprayer assembly was used. This was based on a previously reported design (Takáts *et al.,* 2004) that was modified as previously described (Abbassi-

Ghadi *et al.,* 2015). The distance between the sprayer and inlet capillary was 7 mm, the distance between sprayer and sample surface was 1.5 mm and the distance between inlet capillary and sample surface was <1 mm. The angle between the sprayer and the sample surface was set to 75 °, and the collection angle between inlet capillary and sample surface was 10 °. Methanol/water (95:5 v/v) was used as the electrospray solvent, at a flow rate of 1.5 *µ*L/min, and the spray voltage was set to +4.5 kV. Solvent was delivered using a Dionex Ultimate3000 nLC pump (Sunnyvale, CA, USA). N4.8 Nitrogen (BOC, Guilldford, UK) was used as the nebulizing gas at a pressure of 7 bar. An S-Lens RF level of 75, capillary temperature of 320 °C and mass resolution of 70,000 were employed. Derivatised tissues were analysed in positive ion mode using a mass range of *m/z* 250-1000 and an injection time of 150 ms. All images were recorded with 100 *µ*m spatial resolution resulting in a scan speed of 378.79 *µ*m/s. Compound identities were assigned using exact mass only.

7.3.4 Data and statistical analysis

7.3.4.1 LC-MS/MS data

HGA, tyrosine and nitisinone concentrations were calculated using MassHunter Quantitative Analysis (Build 06.00). A paired t-test was used to compare plasma HGA and tyrosine concentrations at baseline and 1 week using Graphpad Instat (Version 3.10, 2009, CA, USA). A p value <0.05 was deemed significant.

7.3.4.2 Mass spectrometry imaging data

MSI raw data files were initially converted into centroided .mzML files using MSConvert tool (ProteoWizard toolbox Version 3.0.4043) and then further converted into .imzML format using imzML Converter (version 1.3, Race *et al.,* 2012), which were then converted to SCiLS data format for visualization and statistical analysis of the data in SCiLS Lab v2018b. (Bruker Daltonics, Bremen, Germany).

All *m/z* values for monoamine neurotransmitters were extracted with a mass window of less than 0.0052 Da. Derivatisation led to the formation of a [M +

(DPP-TFB)]$^{+}$ ion with no observable [M]$^{2+}$ or [M + Na]$^{+}$ ions. A peak corresponding to the DPP-TFB derivative was observed with a mass shift of +215.0855 to the exact monoisotopic mass. The differences between the theoretical and observed *m/z* values of each derivatised compound was <5 ppm.

Mean intensity and standard deviation of raw *m/z* signal intensity for DPP-TFB derivatives of monoamine neurotransmitters were extracted from tissue regions of interest using SCiLS software (SCiLS Lab Bruker, Version 2018b). To compare signal intensities for extracted *m/z* values for all compounds an unpaired t-test was used; a p value <0.05 was deemed significant.

7.4 Results

7.4.1 LC-MS/MS analysis of plasma homogentisic acid and tyrosine

Plasma HGA concentrations were markedly higher in BALB/c *HGD*$^{-/-}$ mice (AKU mice) at baseline compared to *HGD*$^{-/+}$ mice (non-AKU mice). HGA concentrations in the *HGD*$^{+/-}$ mice were below the lower limit of the measuring interval for the assay. Tyrosine concentrations were similar at baseline between the 3 groups studied; the variation observed between the 3 groups is thought to reflect dietary intake and normal variation in amino acid metabolism.

Treatment of *HGD*$^{-/-}$ mice with nitisinone (mean(±standard deviation) plasma concentration 0.4±(0.094) μmol/L) resulted in a marked decrease in HGA (9-fold, p=0.004) and increase in tyrosine (9-fold, p<0.0001) (Table 7.1).

Genotype	Tyrosine (μmol/L, mean ±SD)			Homogentisic acid (μmol/L, mean ±SD)		
	Baseline	1 week	p value	Baseline	1 week	p value
HGD$^{-/-\ \#}$	112.3 (21.9)	997.8 (112.7)	<0.0001*	197.2 (87.2)	21.6 (4.3)	0.004*
HGD$^{-/-\ \#\#}$	92.8 (29.9)	77.6 (16.2)	0.096	215.7 (168.2)	158.2 (89.8)	0.18
HGD$^{+/-\ \#\#}$	66.5 (7.9)	62.6 (5.8)	0.27	<3.1	<3.1	NS

Table 7.1. Plasma tyrosine and HGA concentrations at baseline and 1 week later in a murine model of AKU. *pvalue <0.05 deemed significant. # – treated with nitisinone (4 mg/L); ## – no treatment; NS – no significance testing performed as concentrations below limit of quantification of assay. Six mice were in each experimental group.

7.4.2 Mass spectrometry imaging of neurotransmitters in murine brain tissue

MS images were successfully generated for all 18 murine brains included in this study in positive polarity. Differences in the appearance of sagittal images (Figure 7.3) is a reflection of the positioning of brains in the mould. All brains were very similar in size and were anatomically symmetrical thus the plane sectioned for all 6 brains in each mould was considered to be comparable and thus ion intensities recorded were considered representative of the plane of tissue sectioned. Figure 7.3 shows representative sagittal MS images of BALB/c mice brains from all 3 treatment groups. As previously described (Shariatgorji *et al.,* 2014; Esteve *et al.,* 2016; Shariatgorji *et al.,* 2016) chemical charge tagging of primary amines with DPP-TFB enabled the detection of DP, 3-MT, noradrenaline (NA), serotonin, tyrosine, tryptophan and tyramine. It is important to note that NA has the same monoisotopic mass as 6-hydroxydopamine (*m/z* 169.0738), thus chemical charge tagging would also theoretically generate a mass shift of +215.0855 to its exact monoisotopic mass. In this study it was assumed the mass shift observed at 384.1575 was NA. This is because it has been hypothesised that 6-hydroxydopamine is a neurotoxic metabolite of DP that is only observed in patients with Parkinson's disease that are treated with L-DOPA (Borah *et al.,* 2012). It is proposed that 6-hydroxydopamine aggravates dopaminergic

neurodegeneration, and it is this that forms the basis of its use in the generation of experimental models of Parkinson's disease (Breese *et al.,* 2005).

Glutamate was also detected, but it is important to note that tissue derivatisation is not required for this and native sections can be used (Shariatgorji *et al.,* 2016). Using a derivatisation approach allowed analysis of all neurotransmitters in a single analytical run while the embedding approach ensured all tissues are treated identically at all times to minimise unequal degradation of neurotransmitters. Tissue drying was found to further increase stability (Swales *et al.,* 2018).

Comparisons of *m/z* ion distribution maps for each compound were made between the 3 groups of mice: (1) BALB/c $HGD^{-/-}$ (n=6, no treatment) *vs*. BALB/c $HGD^{-/-}$ (n=6, treated); (2) BALB/c $HGD^{+/-}$ (n=6, no treatment) *vs*. BALB/c $HGD^{-/-}$ (n=6, treated); (3) BALB/c $HGD^{-/-}$ (n=6, no treatment) *vs*. BALB/c $HGD^{+/-}$ (n=6, no treatment). It is important to note that comparisons made between *m/z* signal intensities represent a relative and not quantitative change in response. In agreement with previous authors (Shariatgorji *et al*., 2014; Shariatgorji *et al*., 2016) the total signal through the brain was used to assess spatial changes in neurotransmitter patterns, with the exception of DP and 3-MT in which only the signal relating to the *striatum* was used. This is because their production is very localised and specific to this anatomical region of the brain. In contrast the other neurotransmitters and related metabolites are distributed more ubiquitously throughout the brain and so the total brain area was used.

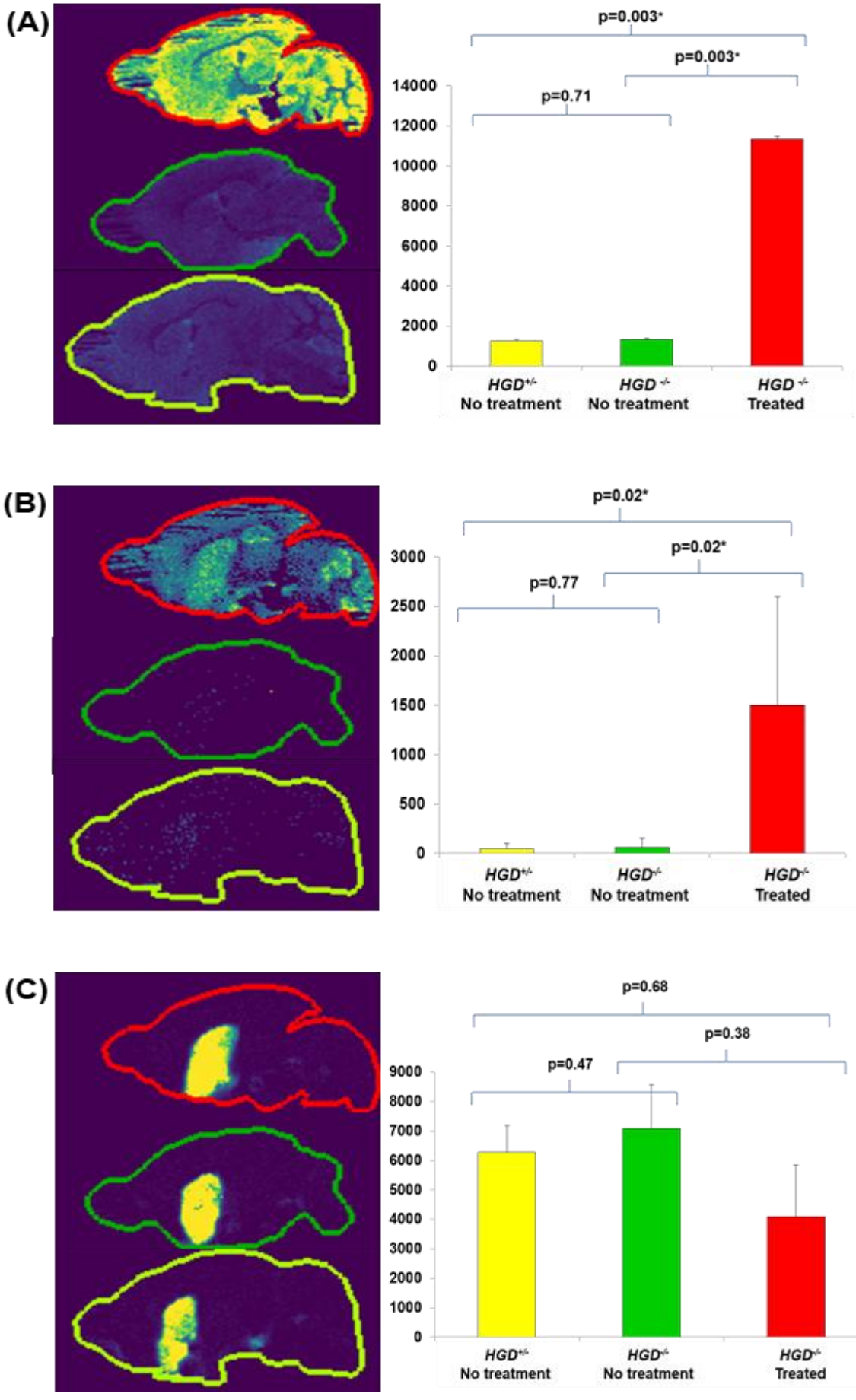
Tyrosine (10-fold, p=0.003; 10 fold, p=0.003; and p=0.71) and tyramine (25 fold, p=0.02; 32 fold, p=0.02; p=0.77) showed significantly more intense signals in mice that received treatment with nitisinone (Figure 7.3 (A) and (B)). Signal intensities for tyrosine and tyramine increased in all regions of the brains studied. Signals were most intense in the *cerebellum*, *cerebral cortex* and *hypothalamus* for tyrosine, and the *striatum* and *cerebellum* for

tyramine. No differences in ion distribution or intensity were observed for the 2 groups of mice that did not receive nitisinone treatment.

DP (*striatum* of brain only, p=0.38, p=0.68, and p=0.47, respectively) and its metabolites 3-MT (*striatum* of brain only, p=0.67, p=0.77, and p=0.94, respectively) and NA (p=0.41, p=0.81, and p=0.54, respectively) did not change significantly following treatment with nitisinone. The intensity of the signal for DP and 3-MT was highest in the *striatum*, *cortical subplate*, and *palladium* (Figure 7.3 (C) and (D)). The intensity of the signal for NA (Figure 7.3 (E)) was more dispersed than that of DP and 3-MT, and was most intense in the *substantia nigra reticulata* and *ventral striatum*.

Serotonin (p=0.54, p=0.78, and p=0.64, respectively) and its precursor amino acid tryptophan (p=0.36, p=0.90, and p=0.27, respectively), and glutamate (p=0.89, p=0.93, and p=0.92, respectively) showed no significant differences between mice that received nitisinone and those that did not. The signal intensity for serotonin was most intense in the *substantia nigra reticulata* and *ventral striatum*, while its precursor tryptophan and glutamate were found in all brain tissues. The latter being most intense in the *cerebral cortex* (Figure 7.3 (F–H)).

All monoamine neurotransmitter compounds and related metabolites in the 2 groups of mice that did not receive nitisinone treatment were not significantly different (Figure 7.3).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HGD -/- No treatment
HGD -/- Treated
(B)
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p=0.02*
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HGD+/- No treatment
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HGD-/- Treated
(C)
p=0.68
p=0.38
p=0.47
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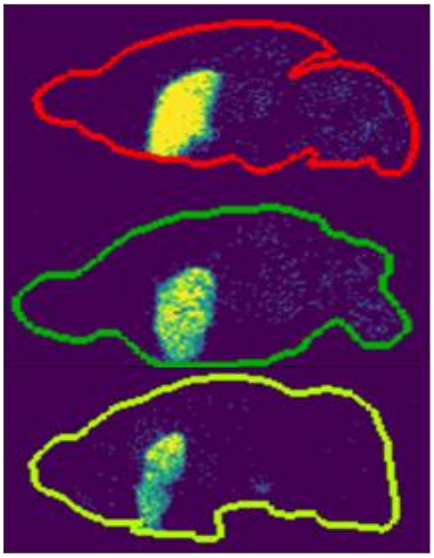
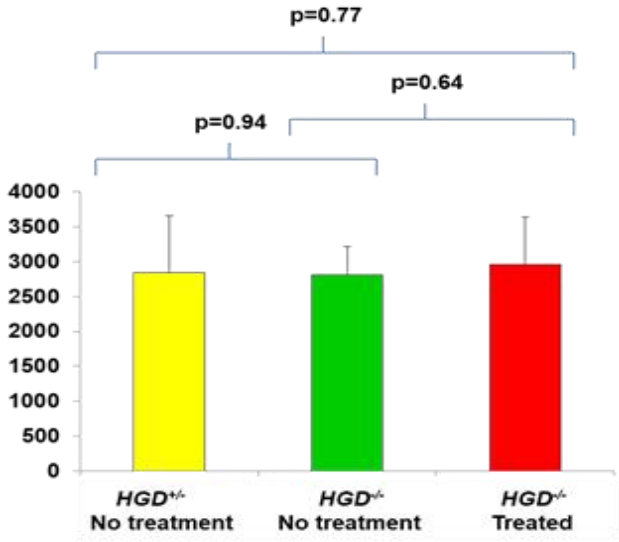
HGD+/- No treatment
HGD-/- No treatment
HGD-/- Treated

(D)

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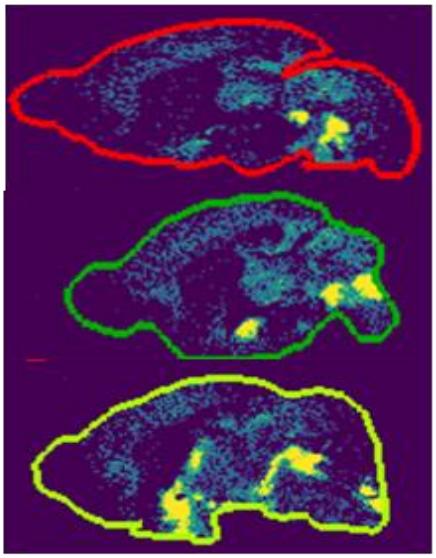
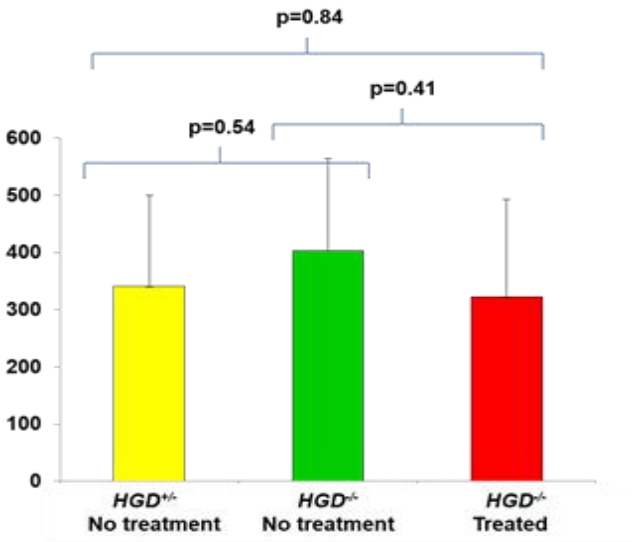
HGD-/- Treated

(E)

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p=0.54
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400
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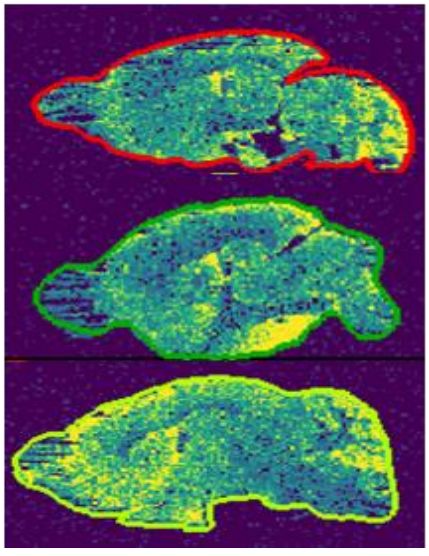
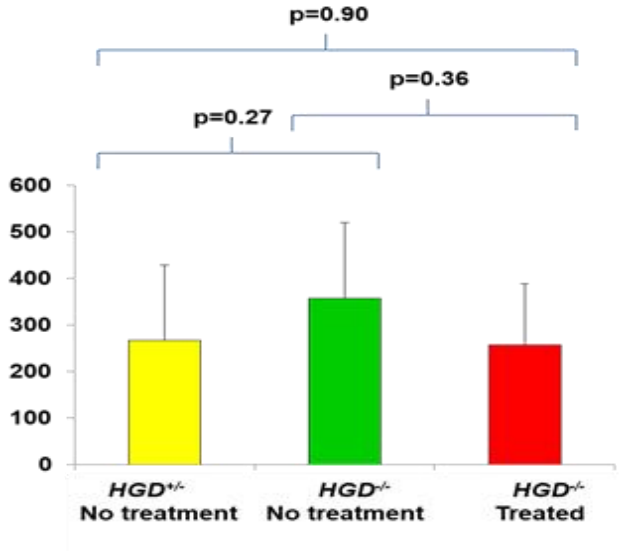
100
0
HGD+/- No treatment
HGD-/- No treatment
HGD-/- Treated

(F)

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HGD-/- No treatment
HGD-/- Treated

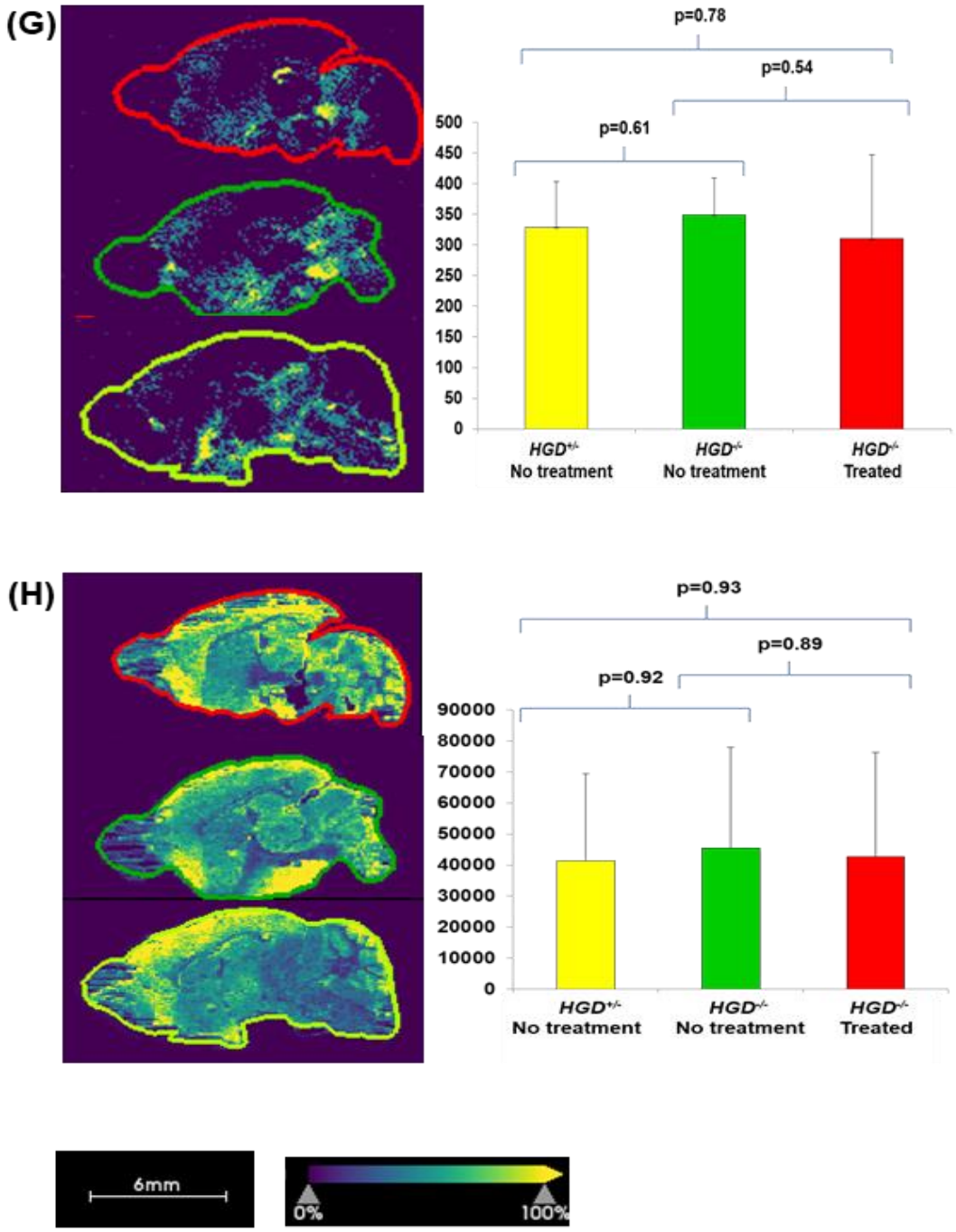

Figure 7.3. Representative mass spectrometry images of sagittal brain tissue sections from the BALB/c mouse. Ion intensity maps of precursor ions of DPP-TFB derivatives acquired in positive ionisation mode (A) Tyrosine *m/z* 396.15814; (B) Tyramine *m/z* 352.16702; (C) DP *m/z* 368.16238; (D) 3-MT *m/z* 382.18013; (E) NA *m/z* 384.15753; (F) Tryptophan *m/z* 419.1753; (G) Serotonin *m/z* 396.15854 and (H) Glutamate *m/z* 362.1364. In each panel Left: sagittal brain sections from BALB/c mice:

HGD$^{-/-}$ mouse treated with nitisinone (red outline); (2) *HGD*$^{-/-}$ mouse no treatment (green outline) and (3) *HGD*$^{+/-}$ no treatment (yellow outline). Right: bar graph: y-axis – mean (± standard deviation) extracted *m/z* ion intensity for metabolite; x-axis – experimental group (n=6 in each group); ion intensity for DP and 3-MT from *striatum* only, not the whole brain section. Scale bar: 6 mm; spatial resolution 100 *μ*m. *p<0.05 deemed significant. Signal intensities are indicated using a viridis colour scale 0-100 % (blue to yellow).

7.5 Discussion

To date the direct consequences of nitisinone induced hypertyrosinaemia on neurotransmitter metabolism in patients with AKU has not been reported. Herein for the first time we report the direct measurement of DPP-TFB derivatives of monoamine neurotransmitters in the brain tissue from a murine model of AKU following treatment with nitisinone, and corresponding plasma concentrations of HGA and tyrosine.

7.5.1 LC-MS/MS analysis of plasma homogentisic acid and tyrosine

Plasma HGA concentrations were markedly higher in BALB/c *HGD*$^{-/-}$ mice (AKU mice) at baseline compared to *HGD*$^{-/+}$ mice (non-AKU mice), this was expected and entirely in keeping with the genotype of the mice studied (Preston *et al.,* 2014).

Treatment of BALB/c *HGD*$^{-/-}$ mice with nitisinone resulted in an expected decrease in HGA and increase in tyrosine. The changes observed occurred because nitisinone is a reversible competitive inhibitor of HPPD (Figure 7.1). These findings confirm that the mice used in this study had the biochemical phenotype one would expect in AKU and that hypertyrosinaemia was observed following treatment with nitisinone.

7.5.2 Mass spectrometry imaging of neurotransmitters in murine brain tissue

Tyrosine and tyramine increased significantly in BALB/c *HGD*$^{-/-}$ mice treated with nitisinone. No changes in tyrosine or tyramine were observed in *HGD*$^{-/-}$ and *HGD*$^{-/+}$ mice that received no treatment. These novel findings support that the changes observed were a consequence of treatment with nitisinone. The increase in tyrosine is likely to be the result of the 9-fold increase in plasma tyrosine, which through facilitated diffusion via the LAT-1 transporter (Mastroberardino *et al.,* 1998) will have resulted in increased transport into the CNS. The large increase in tyramine was an unexpected finding, and is a likely consequence of the action of aromatic acid decarboxylase, which converts tyrosine to tyramine. The physiological role and mechanisms of action of this trace amine remain poorly understood in mammals. Tyramine is often regarded as a by-product of amino acid metabolism with no clear functional relevance (Ledonne *et al.,* 2011).

It is reassuring that DP did not increase itself and is likely to reflect that its synthesis is highly regulated. There is a major and minor route for DP biosynthesis (Meiser *et al.,* 2013). The minor route involves the enzymatic conversion of tyrosine to tyramine via the action of aromatic acid decarboxyalse, tyramine is then converted to DP via cytochrome CYP2D6. The major route is via the enzymatic conversion of tyrosine to L-DOPA and then to DP via the actions of tyrosine hydroxylase and then aromatic acid decarboxylase. In the face of excess tyrosine and tyramine and no change in DP one may postulate that 1 or all of the enzymes mentioned are down regulated to limit DP production when it is not required. A limitation of this study is that enzyme activity was not measured. The increased catabolism of DP to NA via the enzymatic action of DP hydroxylase, or to 3-MT via the enzymatic action of catechol-O-methyltransferase is unlikely as no significant changes were observed in either of these compounds following treatment with nitisinone (Figure 7.3). Importantly this study did not show any changes in serotonin or tryptophan metabolism in *HGD*$^{-/-}$ mice following treatment with nitisinone, confirming that hypertyrosinaemia does not affect serotonin biosynthesis or metabolism in the murine model of AKU studied.

In addition it is reassuring that no significant changes were observed in neurotransmitters or their metabolites at baseline and 1 week in the *HGD*$^{-/-}$ and *HGD*$^{-/+}$ mice that received no treatment, supporting that changes that were observed were a consequence of treatment with nitisinone.

Whilst there have been no reported investigations of the impact of nitisinone induced hypertyrosinaemia on dopaminergic and serotoninergic neurotransmitter metabolism in AKU, the impact of nitisinone induced hypertyrosinaemia on neurotransmitter metabolism has been investigated in a mouse model of phenylketonuria (PKU) (Harding *et al.,* 2014) and HT1 (Hillgartner *et al.,* 2016).

Hillgartner *et al.* (2016) used a similar dose of nitisinone to our group and reported that urinary homovanillic acid (DP metabolite) increased 4-fold following nitisinone, but concluded that this was likely to be a reflection of peripheral breakdown and metabolism did not reflect changes in the CNS. In this study no direct measurements of DP or serotonin were made in brain tissue, but they concluded that slower learning and cognitive difference in the mice studied were caused by HT1 and not by the treatment with nitisinone. We have also reported an increase in a urinary DP metabolite (3-MT) following nitisinone treatment in AKU patients (Davison *et al.,* 2018b; Davison *et al.,* 2018c) and are in agreement that this is likely to reflect a change in peripheral metabolism of catecholamines.

In contrast Harding *et al.* (2014) used a much higher dose of nitisinone (4 mg/mL) than our group (4 mg/L) and as expected this led to hypertyrosinaemia; a 6-fold increase in mean serum tyrosine (256 *μ*mol/L), and a slight but, significant increase in brain tyrosine was observed. This was however far less than what we observed (9-fold increase in mean plasma tyrosine [mean tyrosine 997 *μ*mol/L] and 10-fold increase in tyrosine in the brain tissue) on a thousand times lower dose of nitisinone. This can be explained in large by the metabolic defect in the tyrosine metabolic pathway being proximal to the site of nitisinone action in PKU and distal in AKU (Figure 7.1). In PKU, phenylalanine cannot be converted to tyrosine and thus

far less accumulates as a consequence of nitisinone inhibiting HPPD, in large the observed increase will reflect the accumulation of dietary tyrosine.

Harding *et al.* (2014) also observed that phenylalanine decreased by 44 % and DP increased by 36 % in brain tissue following nitisinone treatment. Herein no significant changes in DP in brain from the AKU mouse were observed following nitisinone. It has been proposed that the lower phenylalanine concentration in brain tissue reduced phenylalanine mediated inhibition of tyrosine hydroxylase, thus enabling DP biosynthesis (Harding *et al.,* 2014). These differences are likely to be accounted for by the fact that Harding *et al.* (2014) performed analysis in brain tissue from a mouse model of PKU and not AKU, which have different metabolic defects.

Importantly in the AKU mouse model there was no significant difference in brain serotonin in *HGD*$^{-/-}$ mice following treatment with nitisinone, or between *HGD*$^{-/-}$ and *HGD*$^{+/-}$ that received no treatment. Serotonin concentrations did not change in PKU animal model following treatment with nitisinone either, but did remain depressed in the PKU mice whether they were treated or not, compared to wild type mice (Harding *et al.,* 2014). It is thought that this was caused by phenylalanine inhibiting the enzyme tryptophan hydroxylase, which regulates the rate limiting step of serotonin metabolism, and cannot be recovered by nitisinone treatment (Harding *et al.,* 2014). Recently Winn *et al.* (2018) provided further support for this hypothesis in a study that showed that while blood phenylalanine reduction corrects DP and serotonin deficiencies in the CNS; it did not recover the activity of tryptophan hydroxylase.

Herein we also showed that glutamate does not change following treatment with nitisinone. The reason for measuring this neurotransmitter was that the glutaminergic neurotransmitter system is known to have a close relationship with dopaminergic and serotoninergic systems (Andreou *et al.,* 2015). Dysregulation of either of the latter may have resulted in a compensatory change in the glutaminergic system.

7.6 Conclusions

Treatment with nitisinone did not affect the ion distribution or intensity of the neurotransmitters DP, NA and serotonin in the AKU mouse brain, suggesting that their concentrations do not change. Neurotransmitter precursor's tyrosine and tyramine were significantly increased following treatment with nitisinone. The significance and fate of these metabolites is unknown and requires further investigation. These findings have critical importance for the use of nitisinone in patients with AKU as they clearly demonstrate that nitisinone does not have a direct effect on monoamine neurotransmitter metabolism in the CNS of mice with AKU. If these findings translate to patients with AKU treated with nitisinone, one can postulate that the hypertyrosinaemia occurring following treatment with nitisinone does not affect monoamine neurotransmitter metabolism and thus is unlikely to result in altered mood or cognition.

7.7 Declaration and acknowledgements

Preparation, analysis and data processing of murine brain tissue by MSI performed by Andrew Davison and Nicole Strittmamtter (AstraZeneca, Cambridge, UK).

Hazel Sutherland (Department of Musculoskeletal Biology I, University of Liverpool, UK) performed the mouse dosing and collection of plasma and brain tissue. The mice in these studies were housed under the licence of George Bou-Gharios (Department of Musculoskeletal Biology I, University of Liverpool, UK).

Thanks to Andrew Hughes for providing support for measurement of tyrosine, nitisinone and HGA in plasma samples and Richard Goodwin (AstraZeneca, Cambridge, UK) for providing the opportunity for me to carry out the work at AstraZeneca.

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Chapter 8

Evaluation of the cerebrospinal fluid metabolome of a murine model of alkaptonuria treated with nitisinone using LC-QTOF-MS

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Unpublished.

8.1 Abstract

Background: Nitisinone induced hypertyrosinaemia is well documented in Alkaptonuria (AKU), and there is uncertainty over whether it may contribute to a decline in cognitive function and or mood by altering neurotransmitter metabolism. The aim of this work was to evaluate the impact of nitisinone on the cerebrospinal fluid (CSF) metabolome in a murine model of AKU, with a view to providing additional insight into metabolic changes that have been previously reported in murine brain tissue using mass spectrometry imaging.

Materials and methods: Experiment 1: 17 CSF samples were collected from BALB/c *HGD*$^{-/-}$ mice (n=8, treated with nitisinone – 4 mg/L and n=9, no treatment). Samples were diluted 1:1 with deionised water and 1 μL was analysed in both polarities using liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS). Raw data were processed using an established accurate mass retention time (AMRT) database. A moderated t-test with Benjamini-Hochberg false-discovery rate adjustment was used to assess changes in metabolite abundance between experimental groups.

Experiment 2: 15 additional CSF samples were collected from BALB/c *HGD*$^{-/-}$ mice (n=8, treated with nitisinone – 4 mg/L, n=7, no treatment). Samples from each treatment group were pooled and 7.5 μL analysed directly using LC-QTOF-MS to assess if a greater number of monoamine metabolites not matched in experiment 1 could be detected. Additionally 9 monoamine metabolite standards were analysed in water and artificial CSF (0.1-4.0 μmol/L) to assess the limit of detection for these compounds using LC-QTOF-MS.

Results: Experiment 1: 5 metabolites had a greater abundance ($\log_2$ fold change (FC) 3.1-13.1, 3/5 were significant $p<0.05$) in the mice that received treatment with nitisinone; 4/5 metabolites related directly to tyrosine metabolism and 1/5 related to tyramine metabolism.

Experiment 2: No additional metabolites were matched using the AMRT database when a larger injection volume of undiluted CSF was analysed. The analysis of monoamine analytical standards showed 5/9 and 1/9 compounds were detected at 0.1 μmol/L and, 4/9 and 7/9 compounds were detected at

0.5 μmol/L in deionsed water and artificial CSF, respectively. One compound was not detected at all in artificial CSF.

Conclusions: Evaluation of the CSF metabolome of a murine model of AKU showed a significant difference in the abundance of a limited number of metabolites. None of these have been reported in CSF from an AKU mouse model previously. Moreover this study confirms that some monoamine metabolites do not appear to be altered following nitisinone therapy, however due to the analytical limitations of LC-QTOF-MS not all metabolites could be evaluated.

8.2 Introduction

The widespread metabolic complication of nitisinone therapy in Alkaptonuria (AKU) (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Sloboda *et al.,* 2019) and hereditary tyrosinaemia type 1 (HT1) (Lindstedt *et al.,* 1992; van Ginkel *et al.,* 2016; Zeybek *et al.,* 2017) is hypertyrosinaemia.

Much of the literature available on hypertyrosinaemia relates to the clinical consequences that have been observed, for example corneal keratopathy and skin rash (Introne *et al.,* 2011; Stewart *et al.,* 2014; Ranganath *et al.,* 2016; Khedr *et al.,* 2018; White *et al.,* 2018). These particular side effects typically resolve when nitisinone is stopped or dosage is modified. A more contentious side effect of hypertyrosinaemia has been linked to alterations in neurotransmitter metabolism, specifically in HT1 where it is estimated that ~35 % children have altered cognitive function following nitisinone therapy (McKiernan *et al*., 2015). The very limited evidence in the literature centres around the direct impact hypertyrosinaemia may have on catecholamine metabolism, specifically dopamine (DP), and the indirect impact it may have on serotonin metabolism (Chapter 1, Figure 1.5), respectively. The reason for this interest is that both DP and serotonin play a pivotal role in cognitive function and mood.

Several mechanisms relating to hypertyrosinaemia have been proposed for altered neurotransmitter metabolism and include: increased transport of tyrosine into the brain and a consequent decrease in the transport of other large neutral aromatic amino acids via the large neutral amino acid transporter 1 (LAT-1) into the brain (*i.e.* tryptophan); increased DP and decreased serotonin in the central nervous system (CNS). It has also been suggested that altered serotonin metabolism may also be due to the direct inhibition of tryptophan hydroxylase (TPH; EC 1.14.16.4) activity (rate limiting step in serotonin biosynthesis) by tyrosine (Chapter 1, Figure 1.5) (Thimm *et al.,* 2011; Hillgartner *et al.,* 2016; Barone *et al.,* 2019).

While these theories are credible the literature is somewhat limited. Thimm *et al.* (2011) reported low concentrations of the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) in cerebrospinal fluid (CSF); however there were only 3 patients in the study. Additionally Hillgartner *et al.,* (2016) showed in a murine model that urinary homovanillic acid (HVA, DP metabolite) increased 4-fold following nitisinone, but concluded that this was likely to be a reflection of peripheral breakdown of catecholamines and did not reflect changes in the CNS. No direct measurements of DP or serotonin were made in brain tissue or CSF, and they concluded that the altered cognition observed was caused by HT1 and not treatment with nitisinone.

More recently Barone *et al.* (2019) demonstrated that human tyrosine hydroxylase isoform 1 and human tryptophan hydroxylase 2, expressed in *Escherichia coli* (BL21), exhibited prominent substrate inhibition kinetics and that enzyme activity decreased at elevated tyrosine levels. This supports the earlier supposition, but in this study no downstream metabolites were measured (*i.e.* DP or serotonin).

There is no direct evidence of altered cognition or central neurotransmitter metabolism in AKU patients with hypertyrosinaemia. In order to assess central neurotransmitter metabolism either CSF or a brain biopsy are required as they directly reflect neurotransmitter metabolism in the CNS. This is an accepted practice in the assessment of neurotransmitter disorders (Pearl *et al.,* 2007), however to obtain these samples would be impractical in patients with AKU owing to their complex musculoskeletal comorbidities and unethical in the absence of clinical evidence of altered cognition or mood. Previous studies in patients with AKU have made assessments of neurotransmitter metabolism in urine and reported increased urinary excretion of the DP metabolite, 3-methoxytyramine (3-MT) in patients receiving nitisinone on a short (4 weeks, 1-8 mg) and long term (2 mg daily, 2 years) basis (Davison *et al.,* 2018b (Chapter 5); Davison *et al.,* 2018c (Chapter 5); Norman *et al.,* 2019a (Chapter 2)). Urinary normetadrenaline (NMA) was also shown to be significantly decreased in the short term study at all doses (1-8 mg) (Davison *et al.,* 2018b (Chapter 5)). Both studies

concluded that the marked increase in 3-MT and decrease in NMA are likely to reflect altered peripheral catecholamine metabolism. This is supported by the finding in the long term study (Davison *et al.,* 2018c (Chapter 6)) that no concurrent change in Beck's depression inventory-II scores were observed.

A recent study in a murine model of AKU using mass spectrometry imaging has reassuringly demonstrated that monoamine metabolite patterns in brain tissue did not change following treatment with nitisinone, with the exception of the trace amine tyramine which increased 25-fold (Davison *et al.,* 2019a (Chapter 7)). This was the first direct evidence that showed DP and serotonin do not change following treatment with nitisinone.

Herein for the first time we report the direct analysis of CSF from a murine model of AKU using liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS) analysis and an established in-house accurate mass retention time (AMRT) database (Norman *et al.,* 2019a; Norman *et al.,* 2019b (Chapter 2); Davison *et al.,* 2019b (Chapter 3)). The rationale for analysing CSF is that it bathes the tissues of the CNS and may give additional insight into changes in neurotransmitters and their respective metabolites and confirm previous findings (Davison *et al.,* 2019a, Chapter 7). Moreover it will allow for changes in the wider CSF metabolome to be assessed following treatment with nitisinone (for a detailed review on the translational utility of CSF in studying neuroscience see de Lange, 2013).

8.3 Materials and methods

8.3.1 Reagents

Water for mobile phases was purified in-house (DIRECT-Q 3UV Millipore water purification system). Methanol, acetonitrile and isopropanol were purchased from Sigma Aldrich (Dorset, UK). Formic acid and ammonium formate were obtained from Biosolve, (Netherlands). All reagents were LC-MS grade. 2 mL amber screw vial and 150 μL glass inserts with polymer feet (maximum recovery vials) were purchased from Agilent (Cheadle, UK) and

artificial CSF from Harvard apparatus (Holliston, MA). All analytical standards were obtained from Sigma (UK) as 1 mg/mL Cerilliant standards. These included catecholamine mixture (contained vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-HIAA, catecholamines mix I (contained adrenaline and noradrenaline (NA), catecholamines mix II (contained NMA and metadrenaline), DP and 3-MT).

8.3.2 Animal experiments and CSF sample collection

A murine model of AKU was used for all experiments as described previously (Preston *et al.,* 2014). Thirty-two BALB/c *HGD* $^{-/-}$ mice (see Figure 8.1 for age and gender) were included in this study, 16 mice were administered nitisinone (4 mg/L) through their drinking water for 1 week, the remaining 16 mice received no nitisinone. All animals were housed in air conditioned rooms (with a 12 h dark/light cycle) at 20 °C and 53 % humidity, with access to food and water *ad libitum* at The University of Liverpool. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

All mice were culled by carbon dioxide asphyxiation and CSF was removed from the *cisterna magna* following the puncture technique of Liu *et al.* (2008), with some modifications. The *cisterna magna* was exposed by dissecting skin and overlaying muscles. Cauterisation was used to dry up any bleeding from surrounding tissues. A pulled glass pipette with an internal diameter of ~0.4 mm was attached to silicone tubing and connected to a 1 mL syringe with a 19 g needle. The tip of the glass pipette was used to puncture the membrane and held still just below the membrane by 1 person. Through a double-headed microscope a second person then used the 1 mL syringe to apply gentle pressure to encourage the CSF to flow up the capillary tube. Only clear CSF samples (*i.e.* non blood stained) were collected. Once collected CSF samples were transferred to clear Eppendorf tubes and stored at -80 °C until analysis. Samples were not acidified. The same glass pipette was used to collect CSF from each animal, and was washed with pure water between each animal.

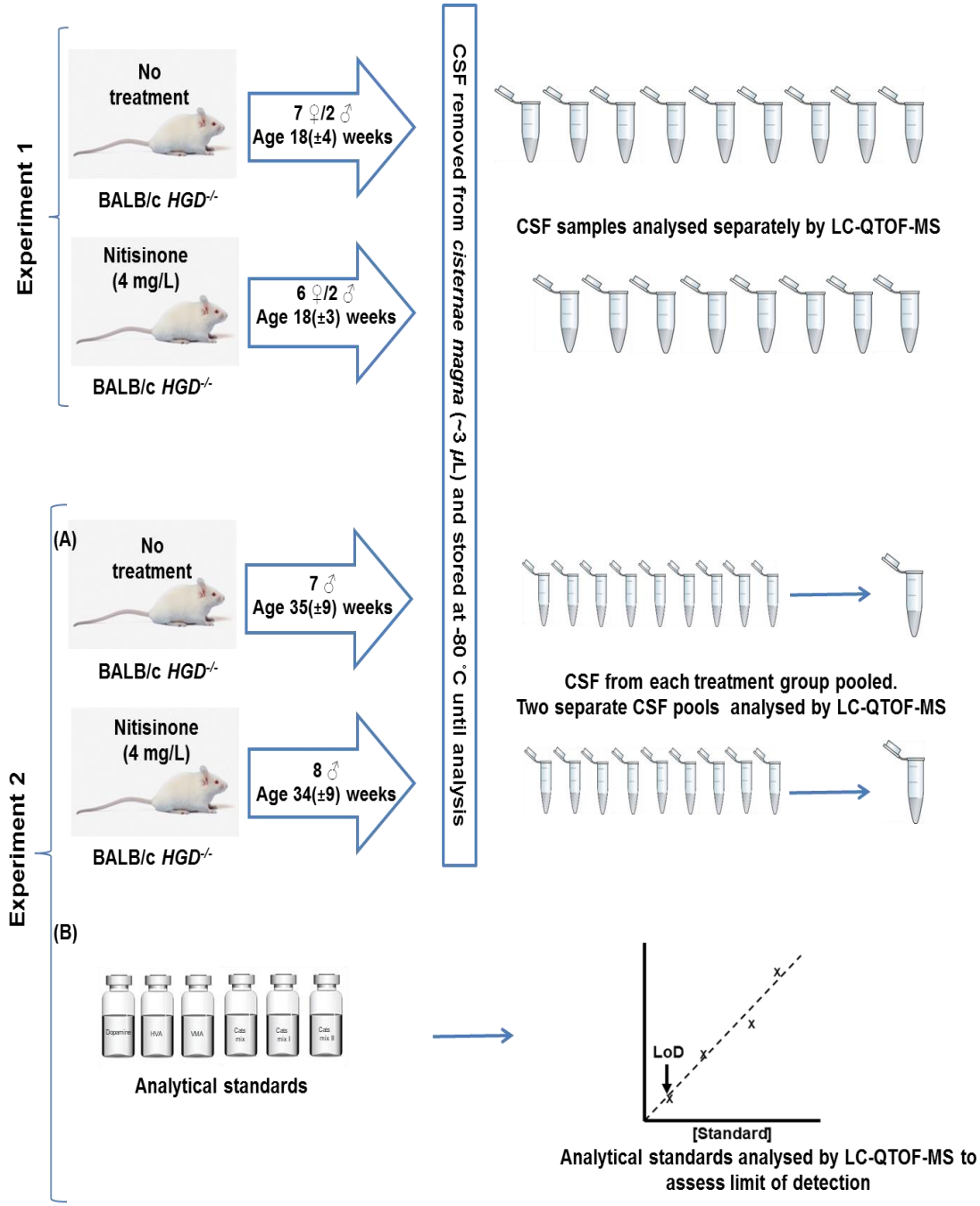

Figure 8.1. Summary of experimental workflow involving murine model of AKU. This includes mouse gender (♂- male; ♀- female) and mean age (±standard deviation); treatment groupings, and sample collection and storage. Experiment 1 – individual CSF sample analysis by LC-QTOF-MS. Experiment 2 – (A) Pooled treatment group CSF sample analysis by LC-QTOF-MS and (B) Analysis of monoamine analytical standards to assess the lowest concentration which can be detected by LC-QTOF-MS.

8.3.4 Murine and quality control sample preparation (Experiment 1)

Murine CSF samples were prepared by diluting 2 μL CSF with 2 μL deionized water (DIRECT-Q 3UV Millipore water purification system). CSF samples were pipetted directly into a 150 μL glass insert with polymer feet, which sat in a 2 mL amber screw vial (Agilent, Cheadle, UK). These vials were used to ensure maximal recovery of sample. Deionised water was also added directly to the glass insert, and mixed with the CSF sample using a pipette. The glass vials were centrifuged at 600 rpm for 10 min to ensure the diluted sample was at the bottom of the vial for sampling. 1 μL of diluted CSF was analysed in negative and positive polarities, respectively.

As CSF sample volume was very limited (~3 μL per mouse) biological group QCs were not prepared. The performance of the analytical run was assessed using system quality control samples that were prepared in artificial CSF (Harvard Apparatus, Holliston, MA). These contained phenylalanine (100 μmol/L), tyrosine (100 μmol/L), nitisinone (10 μmol/L) and succinylacetone (30 μmol/L). Phenylalanine, tyrosine and nitisinone were assessed in positive polarity and succinylacetone in negative polarity. Each analytical run (*i.e.* negative and positive polarities) commenced with 20 replicate injections of the system quality control sample to condition the system.

The order of individual samples was randomised computationally. System QC samples were interspersed throughout the analytical sequence every 6th injection, to assess the analytical system performance. Binary pump pressures and reference ions were also monitored to assess the performance of the LC-QTOF-MS (Figure 8.2).

8.3.5 Murine CSF and monoamine neurotransmitter analytical standard sample preparation (Experiment 2)

8.3.5.1 (A) Murine CSF samples

CSF samples from mice that received nitisinone (n=8) were pooled to make 1 biological pool and CSF from mice that did not receive nitisinone (n=7) were pooled to make a separate biological pool. The rationale for doing this was to generate a large enough CSF sample volume so that CSF could be injected

into the LC-QTOF-MS in a larger volume and without the need for sample dilution, thus improving analytical sensitivity.

Pooled murine CSF samples were not diluted. Each pool consisted of 20 μL and was pipetted directly into a 150 μL glass insert with polymer feet, which sat in a 2 mL amber screw vial (Agilent, Cheadle, UK). 7.5 μL of CSF from each biological pool was analysed in negative and positive polarity, respectively.

8.3.5.2 (B) Monoamine neurotransmitter analytical standards

Analytical standards deemed relevant to catecholamine and serotonin neurotransmitter metabolism were analysed to assess the analytical limit of detection by LC-QTOF-MS (Table 8.1). There is no available literature on the concentration of neurotransmitter metabolites in mice and so concentrations chosen are based on what is reported in humans (*i.e.* adult human CSF 5-HIAA reference range 67-140 nmol/L and HVA reference range 145-324 nmol/L) (Hyland, 2008).

10 μL of each 1 mg/mL Cerilliant standard (total spike volume of 50 μL) was added to 12.45 mL of deionised water (1 in 1250 dilution) to give standard 1 (S1) (Table 8.1). Serial dilutions were then performed to give 3 additional standards S2-S4; concentrations are detailed in Table 8.1. 2 μL of standards S1-S4 were analysed in negative and positive polarity, respectively. This series of dilutions was also performed in artificial CSF to assess the impact of the CSF matrix.

Compound	Monoisotopic mass	Cerilliant standard	S1	S2	S3	S4
Adrenaline	183.2	5458	4366	2018	504	101
Noradrenaline	169.2	5910	4728	2092	523	105
Dopamine	153.2	6528	5222	2195	548	110
Normetadrenaline	183.1	5462	4369	2392	598	120
Metadrenaline	197.1	5073	4058	2183	545	109
3-MT	167.2	5980	4784	2364	591	118
VMA	198.2	5046	4036	2611	652	131
HVA	182.2	5489	4391	2029	507	101
5-HIAA	191.2	5230	4184	2184	546	109

Table 8.1. Summary of monoamine neurotransmitter compounds analysed by LC-QTOF-MS in deionised water and artificial CSF, and the theoretical concentrations. The concentration of all standards is in nmol/L, except Cerilliant standard which is in μmol/L. 5-HIAA – 5-hydroxyindole acetic acid; HVA- homovanillic acid; VMA – vallinylmandellic acid; 3-MT – 3-methoxytyramine.

8.3.6 Analytical method

8.3.6.1 LC-QTOF-MS conditions

LC-QTOF-MS conditions used for the analysis of analytical standards and murine CSF samples were the same as those used for the analysis of urine (Norman *et al.,* 2019a (Chapter 2)) and serum samples (Davison *et al.,* 2019b (Chapter 3).

8.3.6.2 Metabolite identification

Metabolite identification was carried out using an established AMRT database to match chemical entities (Davison *et al.,* 2019b (Chapter 3)). The database included theoretical accurate mass, measured retention time, and empirical formula. It is important to note AMRT data for γ-glutamyl-tyrosine and I-3-L were based on the elution time associated with the theoretical monoisotopic mass of each compound and were not verified using an analytical standard. Data quality control and statistical analysis were performed using the MassHunter software suite (Agilent, Cheadle, UK).

8.3.6.3 Data acquisition and handling parameters

For additional details on data acquisition and handling parameters see Chapter 3 (Section 3.7 Supplementary material).

Data acquired from the analysis of analytical standards were reviewed in Profinder (Build 08.00) as per CSF samples, as the standards analysed were present in the AMRT database. Raw abundance areas for the standards were exported from Profinder as a .csv file and data manipulated in Excel (Microsoft Excel, 2010) to assess linearity. The signal to noise ratios were determined using MassHunter Qualitative software (Build 07.00).

8.3.6.4 Data quality control and statistical analysis

Quality control filters were applied to entities obtained from targeted feature extraction in each profiling experiment. Entities were retained if present in at least 2 samples per experimental group. Data files were then exported from Profinder as '.CEF' files as a whole batch for each profiling experiment and imported to Mass Profiler Professional (MPP) software (Build 14.5, Agilent, Cheadle, UK).

In MPP sample data files were scaled to the median of all samples and chemical entities were filtered based on frequency. Further filtering was then based on variability, and entities were retained if peak area coefficient of variation (CV) remained <25 % across either experimental group. Previously filtering was based on the frequency and variability of entities in biological group QCs across an analytical run (Chapters 2 and 3). Due to the very limited sample volume in this study a different approach was used as it was not possible to make biological group QCs.

Statistical analyses performed in MPP were based on compound signal intensity, expressed as total peak area. Profiles from the experimental group that received nitisinone were compared to the profiles that did not receive nitisinone using a moderated t-test with Benjamini-Hochberg false-discovery rate adjustment. Log_2 fold change (FC) was calculated between each experimental group, and this was based on raw peak area data. Changes

were deemed significant if asymptotic p value was <0.05 and a $\log_2$ FC >2.0. Principal component analysis (PCA) employing a 4 component model was performed on each filtered dataset using MPP.

8.4 Results

8.4.1 Experiment 1 – Quality control and murine CSF data

In the absence of biological group QCs only system QC samples were run every 6th sample. Table 8.2 demonstrates that the analytical run was acceptable as raw peak abundance for all metabolites were <12 % CV, and mean retention time (RT) and ppm error were <0.1 min and <1 ppm, respectively. In addition, reference ion mass calibration and binary pump pressures were very reproducible (Figure 8.2).

Compound	CV of raw area abundance (%)	Mean error from target mass (ppm)	Mean difference from target RT (min)
Tyrosine (100 *µ*mol/L)	8.8	0.69	0.07
Phenylalanine (100 *µ*mol/L)	2.4	0.20	0.06
Nitisinone (10 *µ*mol/L)	11.9	0.26	0.004
Succinylacetone (30 *µ*mol/L)	4.6	0.42	0.02

Table 8.2. Summary of results from the analysis of quality control samples (n=5) throughout analytical runs. RT – retention time; CV – coefficient of variation; ppm – parts per million.

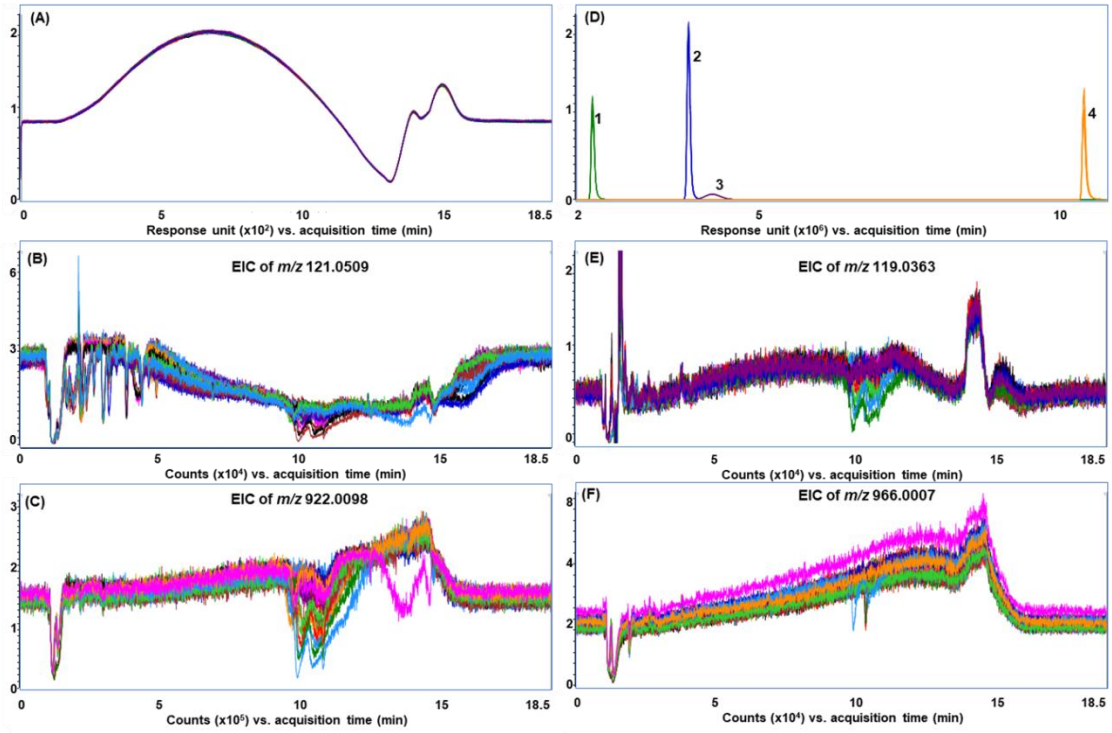

Figure 8.2. Representative data from quality checks performed on data acquired from LC-QTOF-MS analysis of CSF samples in positive and negative polarities. Each analytical sequence consisted of 30 samples.
(A) Overlaid binary pump pressure curves, (B) and (C) Overlaid reference ion signal in positive polarity; (D) Overlaid TIC for quality control samples analysed across the analytical sequence – 1: tyrosine, 2 – phenylalanine, 3 – succinylacetone, 4 – nitisinone (peaks 1, 2 and 4 positive polarity and peak 3 negative polarity); (E) and (F) Overlaid reference ion signal in negative polarity. Extracted ion chromatograms of reference masses were performed to check mass accuracy remained <5 ppm throughout the run. Reference ion signal is present during the analytical runs in both polarities, but shows the greatest suppression of signal at 1-1.5 min. This is near the column void volume where ion suppression is greatest due to elution of none retained entities.

174 and 106 metabolites were aligned across all CSF samples in positive and negative polarities (for matched compounds see Table S8.1) respectively, using an AMRT database. After filtering entities based on their

frequency and variability across the experimental groups 83 and 50 entities were retained from positive and negative polarity profiling experiments, respectively. Of these, 10 and 16 were shown to have a $\log_2$ FC>2.0 in positive and negative polarity, respectively of which 3 entities were significantly different ($p<0.05$), respectively (Figure 8.3).

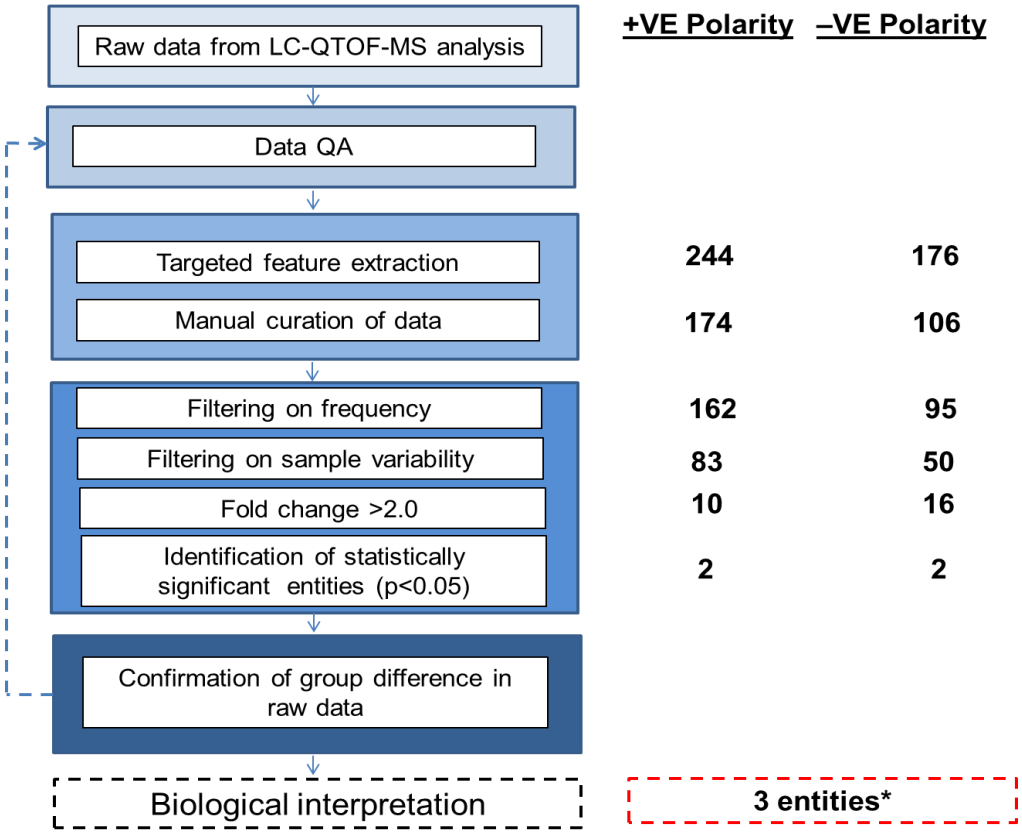

Figure 8.3. Number of accurate mass retention time (AMRT) matches obtained from processing raw LC-QTOF-MS data. AMRT matches generated using the batch targeted feature extraction algorithm (Profinder Build 08.00, Agilent, Cheadle, UK), and subsequent entities retained following quality control filtering and statistical analysis (MPP, Build 14.5) in positive and negative polarity. *1 entity detected in both polarities; QA – quality assessment.

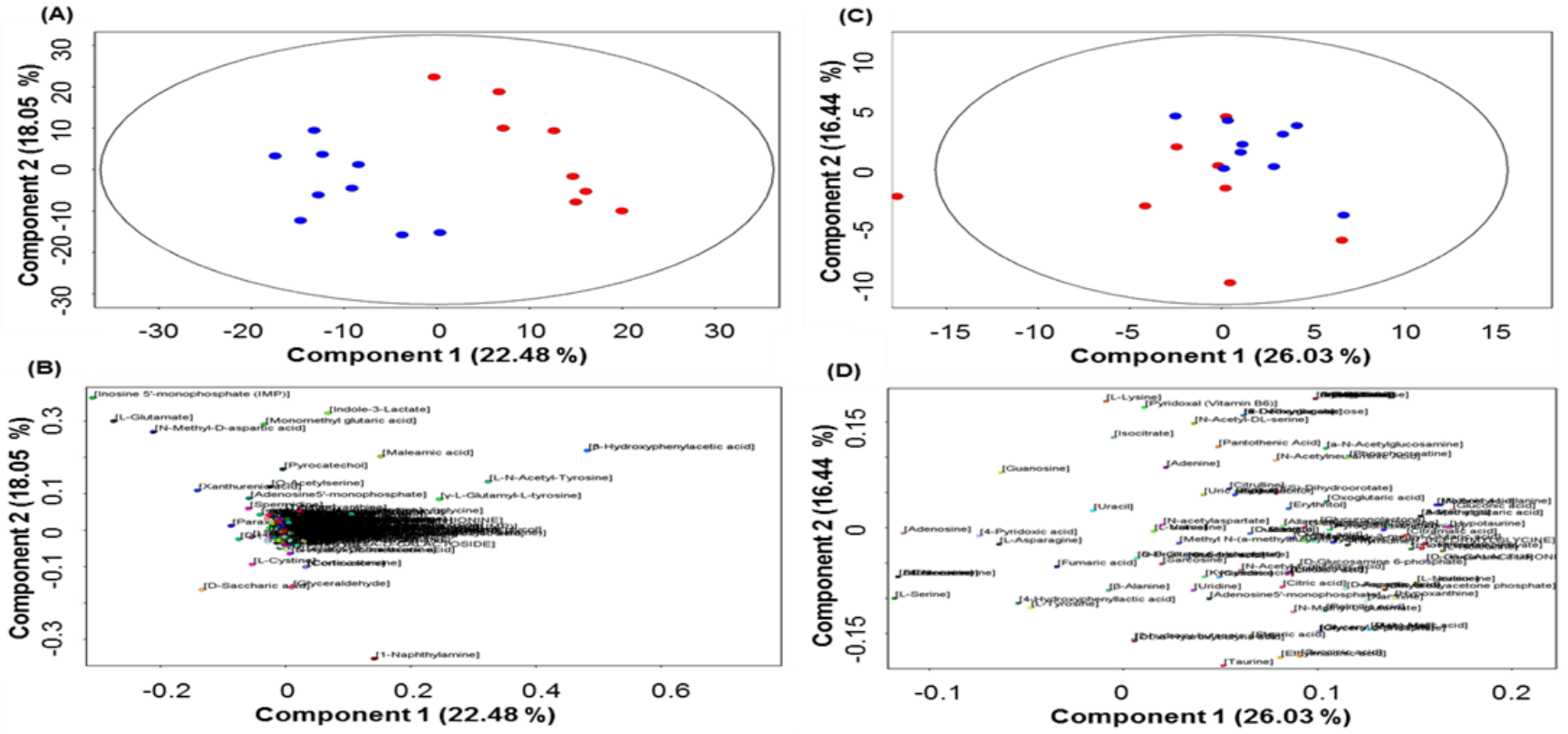

Figure 8.4. Principal component analysis to assess the effect of nitisinone therapy on metabolite profiles in CSF samples collected from AKU mice. Blue circle – no treatment and red circle – nitisinone therapy (4 mg/L, 7 days). PCA plots (A) in positive and (C) negative polarity. Loadings plots (B) and (D) show the respective contributions of individual metabolites to components 1 and 2 in positive and negative polarity, respectively.

Clear separation between the AMRT matched profiles of CSF from untreated and nitisinone treated AKU mice (Figure 8.4) was observed in components 1 and 2 in positive polarity. Negative polarity also showed some separation in components 1 and 2, but was less clear in comparison.

Table 8.3 and Figure 8.5 show compounds in CSF that were significantly different ($p<0.05$ with a $\log_2$ FC >2.0) between the BALB/c *HGD*$^{-/-}$ mice that did and did not receive nitisinone therapy. All 3 entities were related to tyrosine metabolism.

Compound	$\log_2$ FC	Abundance		p value	Metabolic pathway affected
		Up	Down		
p-Hydroxyphenylacetic acid	13.1	√		$1.69E^{-18}$	Tyramine
3-(4-Hydroxyphenyl)lactic acid	4.6	√		$1.58E^{-11}$	Tyrosine
L-Tyrosine*	3.1	√		$5.90E^{-10}$	Tyrosine

Table 8.3. CSF metabolite changes identified post-nitisinone therapy in BALB/c *HGD*$^{-/-}$ mice using an in-house AMRT database. Abundance expressed as $\log_2$ FC, compared to BALB/c *HGD*$^{-/-}$ mice that did not receive nitisinone. $\log_2$ FC included if abundance >2.0 and $p<0.05$. * – increase in abundance observed in positive and negative polarities. Data included represents the lowest $\log_2$ FC, which was observed in negative polarity.

The abundance of γ-L-glutamyl-L-tyrosine and N-acetyl-L-tyrosine was greater in the mice that received nitisinone, the $\log_2$ FC was 6.9 and 5.0, respectively (Figure 8.5). However no p value was calculated for these 2 metabolites as the CV for raw signal abundance was >25 % in both experimental groups. For γ-L-glutamyl-L-tyrosine raw signal abundance had CVs of 26 and 130 % in the mice that were and were not treated with nitisinone, respectively. For N-acetyl-L-tyrosine raw signal abundance had CVs of 31 and 84 % in the mice that were and were not treated with nitisinone, respectively.

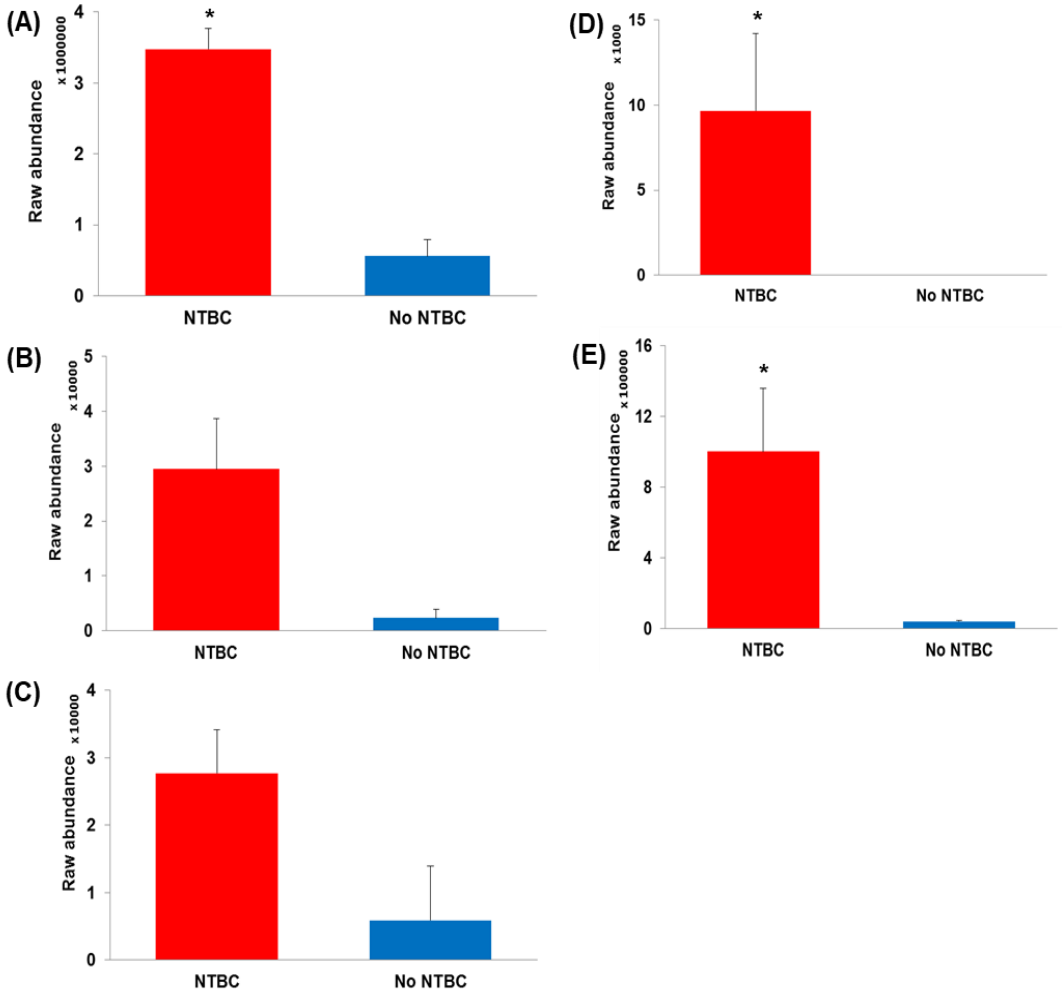

Figure 8.5. Bar graphs showing the mean raw abundances from LC-QTOF-MS analysis for metabolites that were matched to the AMRT database and had a $\log_2$ FC >2.0. Error bars represent +1 standard deviation of the mean raw metabolite abundance (A) Tyrosine; (B) N-Acetyl-L-tyrosine; (C) γ-Glutamyl-tyrosine; (D) p-Hydroxyphenylacetic acid; (E) 3-(4-Hydroxyphenyl)lactic acid. * – indicates significance, $p<0.05$.

In addition to those compounds detailed in Table 8.3 and Figure 8.5, several other metabolites (Figure 8.3) were matched in positive and negative polarities, respectively with a raw abundance CV <25 % in at least 1 experimental group. These metabolites had a $\log_2$ FC <2.0 and no significant differences were observed between the experimental groups. Of particular interest in this study were compounds related to neurotransmitter metabolism

(see Figure 8.6). Reassuringly the large neutral aromatic amino acids (LNAAs) tryptophan and phenylalanine, and the catecholamine metabolites octopamine (trace amine metabolite from tyramine), adrenaline, and 3-methoxy-4-hydroxyphenylglycol (major metabolite of NA) were not significantly different in the 2 experimental groups.

Other metabolites of interest related to catecholamine metabolism included 5-HIAA, dihydroxyphenylacetic acid (DOPAC) and kynurenine. These however had raw signal abundance CVs of 53 and 64 %, 100 and 61 %, and 51 and 86 % in the mice that were and were not treated with nitisinone, respectively. These metabolites were therefore excluded from further statistical analysis. Interestingly homogentisic acid (HGA) was not observed in any of the CSF samples analysed, including those that had not received nitisinone.

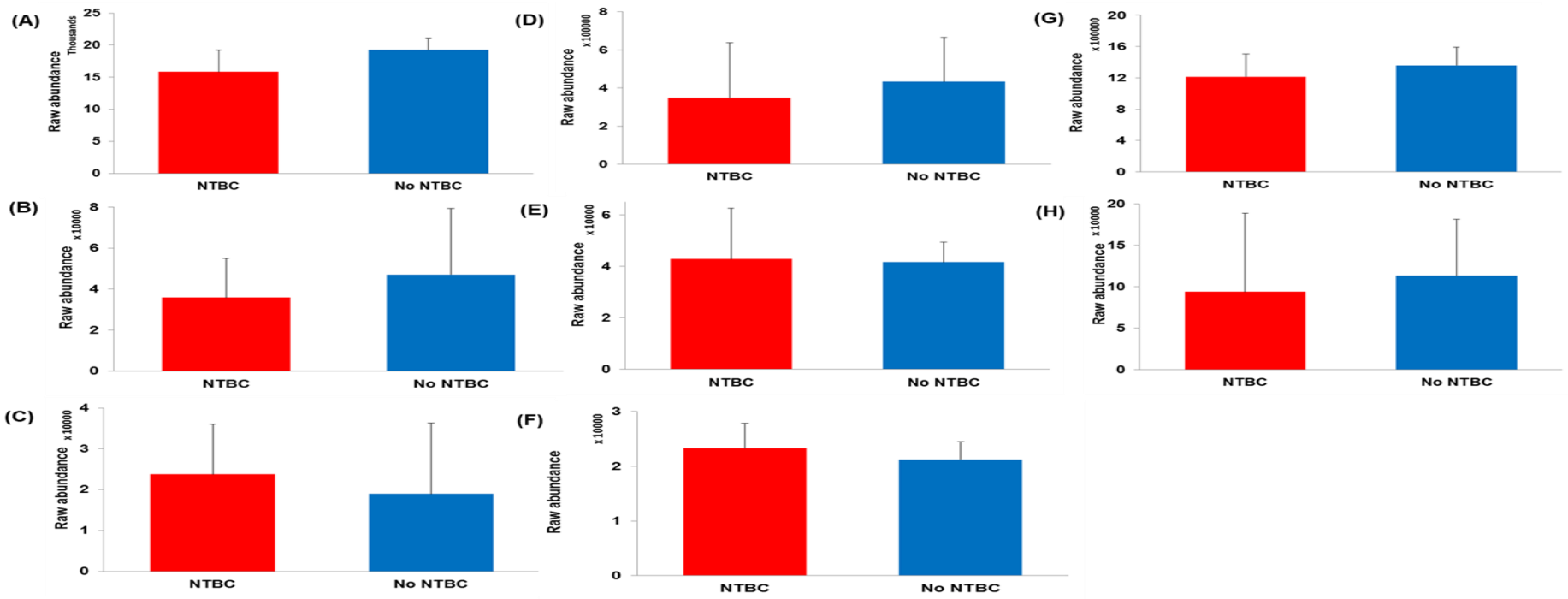

Figure 8.6. Bar graphs showing mean raw abundances from LC-QTOF-MS analysis for AMRT matched metabolites that are important in neurotransmitter metabolism. Error bars represent +1 standard deviation of the mean raw metabolite abundance. The abundance of these metabolites between the 2 experimental groups had a $\log_2$ FC <2.0 and there were no significant differences. (A) Tryptophan, (B) 5-HIAA, (C) Kynurenine, (D) Octopamine, (E) Adrenaline, (F) 3-Methoxy-4-hydroxyphenylglycol, (G) Phenylalanine, (H) DOPAC. It is important to note that 5-HIAA, DOPAC and kynurenine showed large variability within each experimental group (*i.e.* CV >25 %) and were excluded from further statistical analysis.

In this study it was disappointing that the catecholamine metabolites HVA, DP, VMA and tyramine were not matched using the targeted feature extraction in Profinder or through manual extraction of chromatograms in MassHunter Qualitative software (Build 07.00) using the *m/z* of each metabolite in positive and negative polarity, respectively.

8.4.2 Experiment 2 - Analytical standards and murine CSF data

System performance parameters were acceptable as per experiment 1, reference ion signal in negative and positive polarities remained <5 ppm throughout the run and were present during the entire analytical runs in both polarities. Chromatographic binary pump pressures were very reproducible throughout the analytical run.

8.4.2.1 (A) Murine CSF data

176 and 107 compounds were matched in pool 1 and 2 using the AMRT database targeted feature extraction algorithm in positive and negative polarities, respectively. As only 2 samples were analysed, further filtering of data and statistical analysis as in experiment 1 (Figure 8.3) was not possible. The same entities that were observed to have an altered abundance in experiment 1 (Table 8.3) also had an altered abundance in experiment 2, confirming the findings in experiment 1. However the goal of experiment 2 was to match low abundance compounds of interest that were not matched in experiment 1 due to poor sensitivity; it was hoped that MS signal for these compounds would be obtained by not diluting CSF samples and injecting a larger sample volume onto the analytical column. Unfortunately HVA, DP, VMA and tyramine were still not matched in this experiment using the targeted feature extraction in Profinder or through manual extraction of chromatograms in MassHunter Qualitative software. Interestingly, HGA was matched in the 2 samples analysed but did not show a log2 FC >2.0 (Figure 8.6).

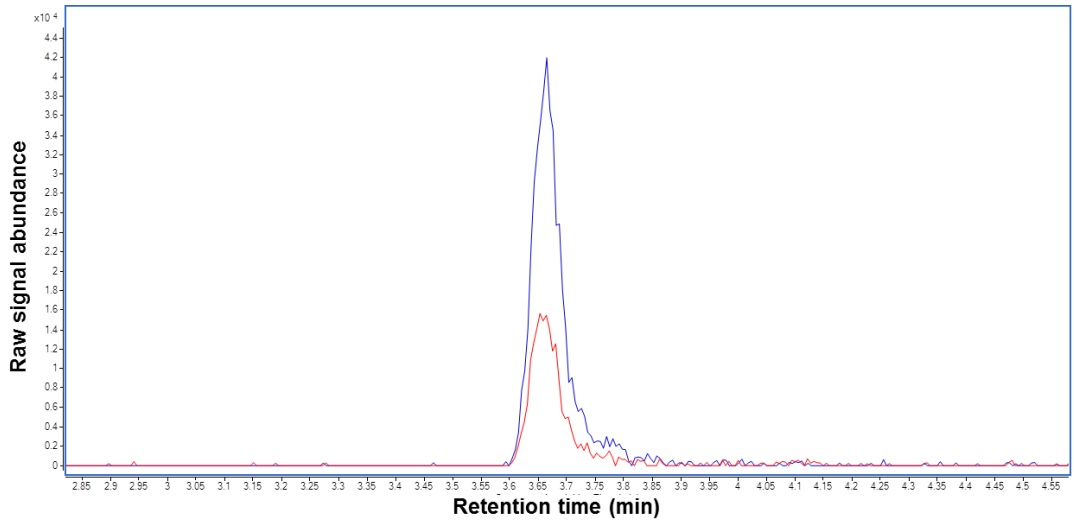

Figure 8.7. Extracted ion chromatogram of HGA in pooled CSF (negative polarity). Blue – mice that received no treatment and red – mice that received nitisinone treatment (ppm error from theoretical mass <10; RT error < 0.15 min from target).

8.4.2.2 (B) Monoamine metabolite standards

Table 8.4 summarises the linear range and signal to noise ratio for the lowest concentration of metabolite detected. All compounds detected showed a good linear response except for NA in deionsed water and DP in artificial CSF.

5/9 and 1/9 compounds were detected at 0.1 μmol/L and, 4/9 and 7/9 compounds were detected at 0.5 μmol/L in deionsed water and artificial CSF, respectively. Interestingly NA was not detected at all in artificial CSF, even at 4.0 μmol/L.

Compound	Deionised water			Artificial CSF		
	Linear Range	R^2	S:N*	Linear range	R^2	S:N*
Adrenaline	0.5-4.0	0.94	60	0.5-4.0	0.96	30
Noradrenaline	0.5-4.0	0.78	13	ND	ND	ND
Dopamine	0.1-4.0	0.98	21	0.5-4.0	0.74	77
Normetadrenaline	0.1-4.0	0.97	23	0.5-4.0	0.95	32
Metadrenaline	0.1-4.0	0.95	21	0.5-4.0	0.94	45
3-MT	0.1-4.0	0.98	50	0.1-4.0	0.96	15
VMA	0.5-4.0	0.99	26	0.5-4.0	0.99	23
HVA	0.5-4.0	0.99	6	0.5-4.0	0.99	6.7
5-HIAA	0.1-4.0	0.96	19	0.5-4.0	0.96	37

Table 8.4. Summary of the linear range for monoamine metabolites measurement by LC-QTOF-MS. All concentrations in μmol/L. ND – not detected; S:N* - signal to noise ratio at lowest standard concentration detected (calculated using Qualitative Analysis software (Build 07.00). 5-HIAA – 5-hydroxyindole acetic acid; HVA- homovanillic acid; VMA – vallinylmandellic acid; 3-MT – 3-methoxytyramine.

8.5 Discussion

Hypertyrosinaemia is a universal biochemical consequence of nitisinone treatment in AKU (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Sloboda *et al.,* 2019) and HT1 (Lindstedt *et al.,* 1992; van Ginkel *et al.,* 2016; Zeybek *et al.,* 2017).

As indicated earlier the rationale for analysing CSF from a murine model of AKU is that it gives a unique insight into the CNS that cannot be gained from the analysis of serum and urine, which provide invaluable yet indirect information.

The study presented herein is the first of its kind to report on the assessment of the CSF metabolome in a murine model of AKU. The reason for the paucity in the literature is likely to reflect the rarity of AKU, but also the small volume of CSF that can be collected from a mouse (~3 μL). The latter presents a significant analytical challenge in terms of sample handling (*e.g.* auto samplers attached to chromatography systems) and also that the metabolites of interest are present at picomolar to nanomolar concentrations. Moreover the technical expertise required to collect non-blood stained CSF from a mouse means the procedure is not frequently performed without some sample contamination

Only 3 metabolites were shown to have significant differences in abundance (log_2 FC >2.0, $p<0.05$) when the metabolite profiles from the 2 treatment groups were compared. All 3 of the metabolites (Figure 8.8 and Table 8.3) were shown to have a greater abundance in the mice that received nitisinone, all of which have been reported to be elevated following treatment with nitisinone in serum (Davison *et al.,* 2019b, Chapter 3) and urine (Norman *et al.,* 2019a, Chapter 2) in patients with AKU, and in the urine of a murine model of AKU (Norman *et al.,* 2019a, Chapter 2).

The increased tyrosine in CSF can be explained by the increased circulating concentrations observed following treatment with nitisinone as a consequence of its action on the HPPD enzyme. This finding is consistent with a previous study that showed nitisinone treatment resulted in significant increases in tyrosine in brain tissue from a murine model of AKU (Davison *et al.,* 2019a, Chapter 7).

The increased abundance of HPLA in the CSF is a curious finding as there are no known mechanisms for its transport into the CNS, unlike tyrosine which goes via the LAT-1 transporter. If HPLA transport occurred via the LAT-1 transporter one could speculate that due to its structural similarity to HGA, the latter would be present in a greater abundance in the CSF, which was not the case in this study as a very low abundance was observed. Additionally if HPLA in CSF was from contamination from the circulation then

HGA would also be present at a higher abundance. The fact that the liver and kidney are the only organs that contain all of the required enzymes for the entire tyrosine metabolic pathway makes the local production of HPLA directly from tyrosine in the CNS unlikely. It is however possible it is produced via an alternative pathway in the brain that is not defined, and requires further investigation. Bernardini *et al.* (2015) has however shown that the *HGD* gene is expressed in murine and human brain tissue, and reported that in the presence of excess HGA cultured neuronal cells produce ochronotic pigment and amyloid. The authors postulated that this may contribute to induction of neurological complications. The implications of the presence of HPLA in the CNS are unknown and also require further investigation. Elevated HPLA has been previously reported in CSF from children with phenylketonuria (PKU) (Antoshechkin *et al.,* 1991) and brain tissue from a rat model of PKU (Sarkissian *et al.,* 2000).

Increased N-acetyl-L-tyrosine and γ-glutamyl-tyrosine in CSF from mice treated with nitisinone was expected based on the observations previously reported in urine (Norman *et al.,* 2019a (Chapter 2)) and serum (Gertsman *et al.,* 2015a; Davison *et al.,* 2019b (Chapter 3)) from AKU patients treated with nitisinone. The large variation in abundance in this study meant the changes observed were not deemed to be statistically significant, however the trend suggests that excess tyrosine is metabolised to N-acetyl-L-tyrosine and γ-glutamyl-tyrosine following treatment with nitisinone. It is presumed that due to their structural similarity to tyrosine both N-acetyl-L-tyrosine and γ-glutamyl-tyrosine are both transported into the CNS via the LAT-1 transporter. Interestingly in CSF the $\log_2$ FC of these metabolites was very similar; in contrast in serum the increase in N-acetyl-L-tyrosine was much greater than that of γ-glutamyl-tyrosine (Davison *et al.,* 2019b, Chapter 3).

The increase in 4-hydroxyphenylacetic acid (HPA) is novel in CSF in the context of AKU as its origin has been reported to be that of gut microbiota (Liu *et al.,* 2016), as previously postulated in serum (Davison *et al.,* 2019b (Chapter 3)). Its presence in CSF cannot be explained by this and is likely to

reflect an increased turnover of tyramine in the brain through oxidative deamination via the action of monoamine oxidase (Figure 8.8). Previous studies have reported low HPA levels in plasma (Yu *et al.,* 1983), urine (Sandler *et al.,* 1979) and CSF (Kobayashi *et al.,* 1982) of depressed patients suggesting a lower turnover of tyramine compared to control subjects.

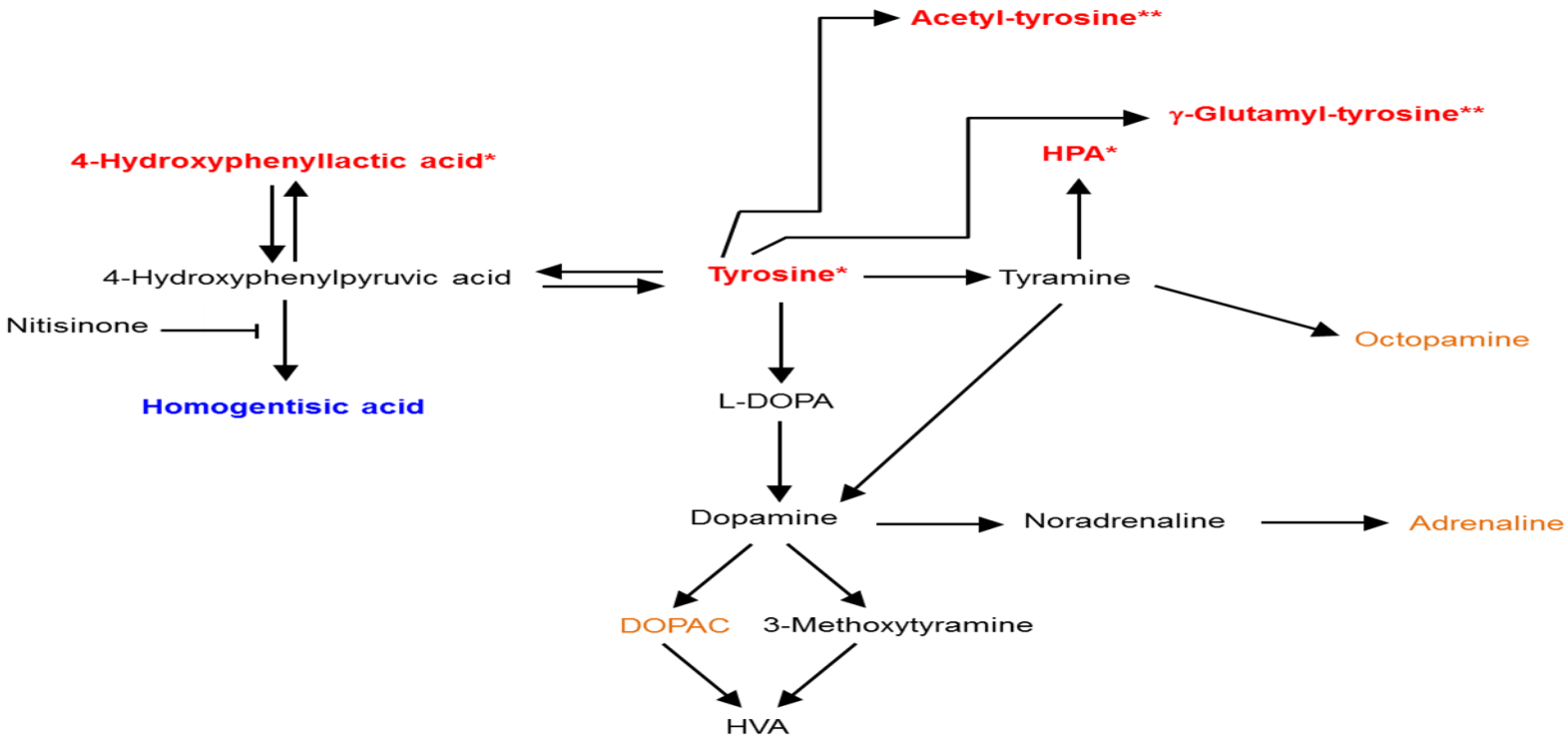

Figure 8.8. Summary of metabolite differences that were observed between *HGD*$^{-/-}$ mice that received no treatment and *HGD*$^{-/-}$ mice that received treatment with nitisinone. Red – compounds that had a higher abundance in CSF from mice treated with nitisinone; blue – compounds that had a lower abundance in CSF from mice treated with nitisinone; orange – metabolites matched in CSF samples, but abundances were not significantly different between the 2 groups of mice; black – compounds not observed in samples. * - $\log_2$FC >2.0, p<0.05; ** - $\log_2$FC >2.0, p>0.05. HVA – homovanillic acid; DOPAC – dihydroxyphenylacetic acid; HPA – 4-hydroxyphenylacetic acid; L-DOPA – L-3,4-dihydroxyphenylalanine.

In a previous study Davison *et al.* (2019a, Chapter 7) reported a 25-fold increase in tyramine in brain tissue from the same murine model of AKU used in this study, herein tyramine was not detected in the CSF analysed in either group of mice. This is likely to reflect that it is present at very low concentrations and that CSF in a mouse is turned over rapidly (*i.e.* total CSF volume of 40 *μ*L is turned over 12–13 times/day (Simon *et al.,* 2016)).

Dourish *et al.* (1982) reported p-tyramine to be present at 5.4 ng/g of mouse brain tissue and that it is metabolised rapidly by its conversion to HPA. In the previous study (Davison *et al.,* 2019a, Chapter 7); monoamine metabolites were derivitised in brain tissue to enhance their detection. Interestingly octopamine, 1 potential product of tyramine metabolism was matched in all CSF samples herein and was not different between the experiment groups. One possible reason for this may be that tyramine in the CNS is preferentially metabolised to HPA as it is not biologically active, unlike tyramine and octopamine (Berry *et al.,* 2007; Lindemann *et al.,* 2008). To this end one may speculate that tyramine is present in the CSF following nitisinone, but is present below the limit of detection for LC-QTOF-MS. Previously tyramine has been regarded as a metabolic dead end, however evidence suggests it, along with other trace amines, can act as neuromodulators for monoamine neurotransmitters, altering the release of and uptake of serotonin, DP and NA (Berry *et al.,* 2007; Lindemann *et al.,* 2008). Several studies have therefore made associations between trace amines and depression, schizophrenia, PKU, Parkinson's disease, Reye's syndrome, Tourette's syndrome, epilepsy, attention deficit hyperactivity disorder, and migraines (Boulton, 1980; Baker *et al.,* 1993; D'Andrea *et al.,* 2004; D'Andrea *et al.,* 2010).

Reassuringly in this study no changes in the abundance of the other large neutral aromatic amino acids (LNAA's) tryptophan and phenylalanine were observed, suggesting that the excess tyrosine observed following nitisinone treatment does not compete for uptake into the CNS via the LAT-1 transporter. This is in contrast to what has been proposed in PKU or HT1, where the excess of phenylalanine and tyrosine, respectively have been proposed to alter neurotransmitter metabolism (Thimm *et al.,* 2011; Thimm *et*

al., 2012; Harding *et al.,* 2014; Hillgartner *et al.,* 2016; Pilotto *et al.,* 2019). It is also unlikely that there is competition for other transporters proposed to be involved in the transport of LNAA's across the blood brain barrier, such as SLCA19 and SLCA15 (O'Mara *et al.,* 2006).

Encouragingly MHPG (NA metabolite), adrenaline, 5-HIAA (serotonin metabolite), DOPAC (DP metabolite) and kynurenine (tryptophan metabolite) were also not significantly altered in nitisinone-treated mice. The latter 3 metabolites did however show large variation in their respective treatment groups so it is important to not over interpret this trend. In contrast in a recent study by Pilotto *et al.* (2019) patients with PKU were shown to have lower 5-HIAA concentrations in CSF compared to controls, suggesting the elevated phenylalanine may contribute to altered neurotransmitter metabolism. Moreover significant negative correlations were observed between CSF 5-HIAA, HVA, and 5-hydroxytyrptophan and phenylalanine. In a small cohort of patients with HT1 lower 5-HIAA in CSF has also been reported (Thimm *et al.,* 2011).

It is uncertain whether the high variation in abundance was a reflection of ion suppression due to the CSF matrix or indeed a limitation of the instrumentation utilised herein (*i.e.* not sensitive enough to measure at such low concentrations). Previous studies have utilised quantitative high pressure liquid chromatography with fluorescence or electrochemical detection (Ormazabal *et al.,* 2005; Akiyama *et al.,* 2017) for measurement of neurotransmitters. Furthermore monoamine metabolites have been derivitised using benzylamine to improve analytical sensitivity (Yoshitake *et al.,* 2006). Targeted quantitative methods utilising liquid chromatography tandem mass spectrometry (LC-MS/MS) have also been reported which have superior analytical specificity and sensitivity (Kovac *et al.,* 2014).

It should be borne in mind that a major limiting factor in this unique study was the very limited sample volume available for analysis. This precluded sample clean up or concentration or indeed application of quantitative LC-MS/MS methodology with the use of an internal standard and matrix matched

calibrators. One approach adopted by previous authors (Ramautar *et al.,* 2012) has been to use sheathless capillary electrophoresis coupled to mass spectrometry which requires nanolitre injection volumes.

Disappointingly DP, and its major metabolites 3-MT and HVA, and the major metabolite of adrenaline VMA were not detected in the samples analysed. The reasons for this are not known as these metabolites are likely to be present at similar concentrations to 5-HIAA as they are in humans and have a short half life (Hyland, 2008). The previous discussion around ion suppression from the CSF matrix and the analytical capability of the LC-QTOF-MS are equally relevant for these metabolites. Additionally the analysis of a small volume of diluted sample may have impacted on their measurement. As samples were frozen at -80 °C immediately after collection it is unlikely that this is a reflection of metabolite degradation.

An additional consideration with analysis of monoamine metabolites using the Agilent 6550 LC-QTOF-MS is the geometry of the hardware, specifically this model has 2 ion funnels which may predispose more 'fragile' metabolites to break up impacting on sensitivity and reproducibility.

To investigate the impact of diluting CSF samples on the detection of metabolites additional experiments were performed (experiment 2), in which pooled CSF samples from respective treatment groups were analysed (Figure 8.1). This meant a larger sample volume was available for analysis and samples were not diluted. Disappointingly this strategy did not improve the detection of 3-MT, HVA, tyramine or VMA meaning that the impact of hypertyrosinaemia on DP metabolism in CSF could not be fully evaluated. It did however show HGA was present at low abundance in CSF and decreased following treatment with nitisinone (Figure 8.6).

To assess whether the inability to detect some monoamine metabolites was related to the sensitivity of the LC-QTOF-MS method used, monoamine analytical standards were analysed in water and artificial CSF at appropriate concentrations. Tyramine was not analysed as it is known to be present at

low picomolar concentrations that a LC-QTOF-MS cannot measure without sample concentration or clean-up (experiment 2). Data showed that in a water matrix all monoamine metabolites studied were detected at 0.1 μmol/L (5/9 standards) or 0.5 μmol/L, this included HVA, 3-MT and VMA. In contrast, analysis in artificial CSF showed that 7/9 standards were detected at 0.5 μmol/L, and one standard (NA) was not detected at all. Interestingly 3-MT was detected at 0.1 μmol/L in analytical standards in deionised water and artificial CSF, but not mouse CSF. The reason for this is not known and requires further investigation. Together these data provide support that the use of LC-QTOF-MS for the analysis of monoamine neurotransmitter in CSF without sample clean up or concentration can provide valuable but somewhat limited information. Further work is required to assess the impact of hypertyrosinaemia on both DP and serotonin related metabolites in CSF using a targeted quantitative method like LC-MS/MS. This will however not be without its own challenges due to the limited volume of CSF available from a mouse. An alternative animal model could also be considered, for example the rat where larger volumes of CSF can be obtained for analysis as total CSF volume is estimated to be 250 μL (Bass *et al.,* 1973).

8.6 Conclusions

This is the first study to report the impact of treatment with nitisinone on the CSF metabolome, and specifically on monoamine neurotransmitter metabolites using LC-QTOF-MS and an established AMRT database. This study shows that a small number of tyrosine related metabolites had a greater abundance in mice treated with nitisinone. Further work is required to understand the significance of these findings and the mechanisms by which the altered metabolite abundances occur.

Moreover this study showed that LC-QTOF-MS was not able to fully evaluate all neurotransmitter metabolites of interest due to the limit of detection for these metabolites using this analytical approach.

8.7 Supplementary material

Compound	Polarity	
	Positive	Negative
(-)-Adrenaline	x	
(S)-Dihydroorotate		x
10-Hydroxycapric acid	x	
12-Hydroxydodecanoic acid	x	
1-Aminocyclopropane-1-carboxylic acid	x	
1-methylhistidine	x	
1-Naphthylamine	x	
2-Amino-1-phenylethanol	x	
2-Aminoisobutyric acid	x	
2-Deoxy-D-ribose	x	
2-Deoxyglucose	x	x
2-Hydroxy-butanoic acid		x
2-Methylglutaric acid	x	x
3,4-Dihydroxybenzoic acid	x	
3-Amino-4-hydroxybenzoic acid	x	
3-Carboxypropyl trimethylammonium	x	
3-Hydroxy-3-methyl-glutaric acid	x	x
3-Hydroxyphenylacetic acid	x	
3-Methoxy-4-hydroxyphenylglycol	x	
3-Methyladenine	x	
3-Methylglutaric acid	x	x
3-Ureidopropionic acid	x	
4-Acetamidobutanoate	x	
4-Hydroxy-L-phenylglycine	x	
3-(4-Hydroxyphenyl)lactic acid		x
4-Pyridoxic acid	x	x
5′-Deoxy-5′-(methylthio)adenosine	x	
5-Hydroxyindole acetic acid	x	
6-Deoxy-L-galactose	x	x
Adenine	x	x
Adenosine	x	x
Adenosine-5'-monophosphate	x	x
Adipic acid	x	x
AHBA	x	
Allantoin		x
Allose	x	x
Allothreonine	x	x
α-L-Rhamnose		x
α-N-Acetylglucosamine	x	x

Caffeine	x	
Caldine	x	
Carnitine	x	
Carnosine	x	
Chenodeoxycholic acid	x	
Citramalic acid		x
Citric acid		x
Citrulline	x	x
Cortexolone	x	
Corticosterone	x	
Creatine	x	
Creatinine	x	
Cytidine	x	x
Cytosine	x	
D-(-)-Lyxose	x	
D-(+)-Cellobiose	x	
D-(+)-Galacturonic acid		x
D-(+)-Malic acid	x	x
D-(+)-Mannose	x	x
D-(+)-Xylose	x	
D-Alanine	x	
D-Aspartic acid	x	x
Deoxycholic acid	x	
Deoxycytidine	x	
Deoxyguanosine	x	
Deoxyuridine monophosphate	x	
D-Fructose-6-phosphate	x	x
D-Galactose	x	x
D-Glucosamine-6-phosphate	x	x
D-Glucose 6-phosphate	x	
D-Glucuronic acid		x
Diacetyl	x	
Diethanolamine	x	
Dihydrouracil	x	
Dihydroxyacetone phosphate		x
Dihydroxyphenylacetic acid	x	
DL-3-Amino-isobutanoic acid	x	
DL-α-Hydroxybutyric acid		x
DL-Homoserine	x	x
DL-pipecolic acid	x	
D-Mannitol	x	x
D-Mannose 6-phosphate	x	
D-Ornithine	x	

D-Psicose	x	x
D-Ribose	x	
D-Saccharic acid	x	
D-Sorbitol		x
D-Tagatose		x
D-Tryptophan	x	x
Dulcitol	x	x
Elaidic acid		x
Erythritol	x	x
Ethylmalonic acid	x	x
Fumaric acid		x
γ-Aminobutryic acid	x	
γ-L-Glutamyl-L-tyrosine	x	
Gluconic acid		x
Glucuronolactone		x
Glutamine	x	x
Glyceraldehyde	x	
Glycerol-2-phosphate	x	x
Glyceryl phosphate	x	x
Glyoxylic acid		x
Guanine	x	
Guanosine		x
Hippuric acid	x	
Histamine	x	
Hypotaurine		x
Hypoxanthine	x	x
Indole-3-lactate	x	
Inosine	x	x
Inosine-5'-monophosphate	x	
Isocitrate	x	x
Ketoleucine	x	
Kynurenic acid		x
L-(-)-Arabitol	x	x
L-(-)Sorbose		x
L-2-Aminoadipic acid	x	
L-Alanine	x	
L-Anserine	x	
L-Arginine	x	
L-Asparagine	x	x
L-Aspartic Acid	x	x
L-Cystine	x	
L-Glutamate	x	
L-Histidine	x	

Linoleic acid		x
L-Isoleucine	x	x
L-Leucine	x	x
L-Lysine	x	x
L-Methionine	x	x
L-N-Acetyl-tyrosine	x	
L-Norleucine	x	x
L-Norvaline	x	
L-Norvaline		x
L-Ornithine	x	
L-Phenylalanine	x	x
L-Pipecolic acid	x	
L-Proline	x	
L-Serine	x	x
L-Threonine	x	x
L-Tryptophan	x	x
L-Tyrosine	x	x
L-Valine	x	x
Maleamic acid	x	
Malic acid	x	x
Malonic acid		x
Methyl N-(α-methylbutyryl)glycine	x	x
Methyl-β-D-galactoside	x	
Monomethyl glutaric acid	x	
Myoinositol		x
N-α-Acetyl-L-asparagine	x	
N-Acetylaspartate	x	x
N-Acetyl-D-galactosamine	x	
N-Acetyl-DL-methionine	x	
N-Acetyl-DL-serine	x	x
N-Acetyl-L-alanine	x	x
N-Acetyl-L-glutamic acid	x	x
N-Acetylneuraminic acid	x	x
N-Acetyl-β-D-mannosamine	x	
N-Formylglycine		x
N-Formylmethionine	x	
Niacinamide	x	
N-Methyl-D-aspartic acid	x	
N-Methylhistamine	x	
N-Methyl-L-glutamate	x	x
Nε,Nε,Nε-Trimethyllysine	x	
O-Acetylserine	x	
Octopamine	x	

O-Phosphorylethanolamine	x	
Oxoglutaric acid		x
Palmitic acid		x
Pantothenic acid	x	x
Paraxanthine	x	
Petroselinic acid	x	
Phosphocreatine	x	x
Phosphoenol pyruvate		x
4-Hydroxyphenylacetic acid	x	x
Pimelic acid	x	
Pyridoxamine	x	
Pyrocatechol	x	
Pyroglutamic acid	x	x
Quinic acid		x
R-(-)-Mandelic acid	x	
Ribitol	x	x
Riboflavin	x	
S-(5-Adenosyl)-L-methionine	x	
S-Adenosylhomocysteine	x	
Salsolinol	x	
Sarcosine	x	x
Spermidine	x	
Stearic acid		x
Suberic acid	x	x
Succinic acid		x
Taurine	x	x
Thiamine monophosphate	x	
Thymidine	x	x
Trigonelline	x	
Uracil	x	x
Uric acid		x
Uridine	x	x
Urocanic acid	x	
Xanthine	x	x
Xanthosine	x	
Xanthurenic acid	x	
Xylitol	x	x
α-D-Galactose-1-phosphate	x	
α-D-Glucose	x	x
α-D-Glucose-1-phosphate	x	x
β-Alanine		x

Supplementary Table S8.1. Summary of metabolites that were aligned and matched across samples in each experimental group. Metabolites were aligned using Profinder software (Build 08.00, Agilent, Cheadle, UK); a targeted feature extraction was used to align profiled experimental data with data in the accurate mass and retention time database containing 469 intermediary metabolites. Metabolites aligned and matched in both negative (n=106) and positive (n=174) polarities are detailed. Feature extraction employed a window of theoretical accurate mass ± 10 ppm and retention time ± 0.30 min. Allowed species were: H^+, Na^+ and NH_4^+ for positive polarity; and H^- and CHO_2^- for negative polarity. Dimers were allowed for both polarities. Charge state range was 1-2.

8.8 Declaration and acknowledgements

Preparation, analysis and data processing of CSF samples performed by Andrew Davison.

Hazel Sutherland (Department of Musculoskeletal Biology I, University of Liverpool, UK) performed the mouse dosing and collection of CSF samples. The mice in these studies were housed under the licence of George Bou-Gharios (Department of Musculoskeletal Biology I, University of Liverpool, UK).

Part IV

Overall Conclusions, References and Appendix 1

Chapter 9

Overall Conclusions

The body of work presented herein is the most extensive evaluation of the impact of nitisinone treatment on the metabolome in humans and mice with Alkaptonuria (AKU) using mass spectrometry based techniques.

In reference to the original aims of this work an analytical strategy has been established and applied for the evaluation of (i) the serum and urine metabolome of patients with AKU and (ii) the urine and cerebrospinal fluid (CSF) metabolome of mice with AKU, following treatment with nitisinone. Moreover the work presented herein addresses the more specific question regarding the impact of nitisinone induced hypertyrosinaemia on neurotransmitter metabolism through (i) the quantitative measurement of urinary monoamine neurotransmitter metabolites and their related serum amino acids in AKU patients, and (ii) the qualitative assessment of monoamine neurotransmitters in CSF and brain tissue from a murine model of AKU.

Key findings relating to the wider impact of treatment with nitisinone on tyrosine metabolism (Figure 9.1), tryptophan metabolism (Figure 9.2) and oxidative stress (Figure 9.3) are presented, integrating findings from the analysis of serum and urine from AKU patients and, urine, plasma, CSF and brain tissue from AKU mice. One of the major observations in the studies performed is how a number of metabolic pathways appear to be affected as a consequence of nitisinone treatment that are not classically associated with AKU and or its treatment with nitisinone. A number of changes in the abundance of metabolites relate to the hypertyrosinaemia observed following the specific inhibition of 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C.1.13.11.27), the enzyme proximal to the defect in homogentisate 1,2-dioxygenase observed in AKU.

Nitisinone-induced hypertyrosinaemia has been reported extensively in AKU (Phornphutkul *et al.,* 2002; Suwannarat *et al.,* 2005; Introne *et al.,* 2011; Ranganath *et al.,* 2016; Milan *et al.,* 2017; Davison *et al.,* 2018a; Sloboda *et al.,* 2019) and has been confirmed herein in urine and serum from AKU patients, and plasma, urine, CSF and brain tissue from mice. This finding is

highly novel in mouse CSF and brain tissue. Moreover, with the exception of human serum (Gertsman *et al.,* 2015a; Gertsman *et al.,* 2015b), this is the first study to report this using high resolution mass spectrometry and nuclear magnetic resonance spectroscopy.

Moreover the work presented demonstrated a significant increase in 3-(4-hydroxyphenyl)pyruvic acid (HPPA) (urine only) and 3-(4-hydroxyphenyl)lactic acid (HPLA) in AKU patients following treatment with nitisinone (Figure 9.1). This confirms previous findings (Gertsman *et al.,* 2015b; Milan *et al.,* 2019), but adds to the picture because for the first time changes were also observed in urine and CSF (HPLA only) from AKU mice, which is unique to the studies presented herein. The reason for not observing HPPA in serum is uncertain and requires further investigation as it has been reported to increase following treatment with nitisinone (Gertsman *et al.,* 2015b; Milan *et al.,* 2019). One may postulate that sample preparation and or analytical conditions may have impaired the ability to detect in HPPA. Moreover it is uncertain whether this metabolite is labile and thus may degrade.

Important findings in relation to the alternative fate of tyrosine observed following nitisinone therapy include an increase in the abundance of N-acetyl-L-tyrosine and γ-glutamyl-tyrosine in serum and urine from AKU patients. These findings also confirm what has been previously reported by Gertsman *et al.* (2015a), but additionally demonstrate these changes occur in urine and CSF from AKU mice. Novel metabolites relating to tyrosine metabolism were also observed, including an increase in urinary coumarate. Together these metabolites demonstrate an altered route of metabolism to facilite the elimination or indeed detoxification of tyrosine in the body, beyond the conventional pathways for its catabolism and transformation to other biologically important molecules (*i.e.* thyroid hormones, catecholamines, melanin).

Biochemical observations in urine from the studies in mouse and human subjects confirm that in fact peripheral catecholamine metabolism is affected

following treatment with nitisnone, specifically dopamine and noradrenaline metabolism (Figure 9.1). Interestingly all metabolites that were altered following treatment with nitisinone had a decreased abundance (Figure 9.1), except urinary tyramine, 3-methoxytyramine (3-MT) and mandelic acid, the latter also had an increased abundance in serum. The increase in urinary 3-MT was observed in all AKU patients that received a daily dose of nitisinone (1-8 mg) in the suitability of nitisinone in AKU 1 (SONIA-1) trial and those patients that attend the National Centre for AKU (NAC), the latter cohort also showed an increase in urinary tyramine. Interestingly in the NAC cohort where patients had 2 mg of nitisinone every other day (*i.e.* up to 3 months) the increase in 3-MT was not observed. Changes in 3-MT and tyramine were not observed in serum samples collected from patients with AKU, or CSF samples collected from AKU mice. The exact mechanism for these urinary changes is not known.

One may postulate the observed decrease in normetadrenaline, in the short term SONIA-1 dosing study may be a consequence of reduced sympathetic nerve excitation as subjects were less depressed or anxious, as they are on treatment for AKU. Previous studies (Roy *et al.,*1986a; Roy *et al.,* 1986b; Grossman *et al.,* 1999; Hughes *et al.,* 2004) have evaluated the urinary concentration of noradrenaline and adrenaline in subjects with depression, demonstrating that urinary concentrations of noradrenaline and adrenaline were significantly higher in subjects with depression compared to controls. This decrease in normetadrenaline was however not observed in patients attending the NAC who had metadrenalines evaluated after 3 months. The reason for this is unknown, but may relate to the body adapting to the long term use of nitisinone such that this change is no longer observed. Moreover this was a larger cohort of AKU patients.

The marked increase in urinary 3-MT and tyramine, and decrease in the major dopamine metabolite homovanillic acid (HVA) suggest there is an altered route of dopamine metabolism, the course of which was not elucidated in this study. The increase in mandelic acid in serum and urine, a metabolite of vanillylmandelic acid, supports the supposition that there is an

altered route of monoamine metabolism. The significance of the latter is unknown, but has been reported previously in patients with phenylketonuria (Rampini *et al.,* 1974).

It is widely accepted that the metabolism of catecholamine neurotransmitters is complex and they have multiple origins including the sympathetic nerves, adrenal medulla, brain and mesenteric organs (Eisenhofer *et al.,* 2004), and are affected by dietary intake (de Jong, 2009). Importantly it has been demonstrated that the concentration of monoamine neurotransmitters in urine are dependent on plasma concentration and uptake via organic cation transporters in the kidney (Eisenhofer *et al.,* 1996; Graefe *et al.,* 1997), thus one may postulate that the changes observed in urine relate to alterations in the renal transport, and the intrarenal metabolism of tyrosine following treatment with nitisinone, and its respective metabolism to catecholamine metabolites.

Beck's depression inventory II (BDI-II) data included in this study support that central metabolism of monoamine metabolites is not altered in patients following treatment with nitisinone. This clinical assessment of mood is a widely accepted tool in clinical practice, and effectively is a phenotypic reflection of dopamine and serotonin neurotransmitter metabolism. Whilst BDI-II scores were marginally increased in patients followed for 24 months post treatment the majority of patients remained in the minimal depression catergory. Moreover these data were not correlated with urinary monoamine metabolites.

Encouragingly animal studies did not demonstrate any changes in catecholamine neurotransmitter patterns in brain tissue following treatment with nitisinone. These studies did however reveal that the trace amine tyramine increased following nitisinone treatment. The significance of this novel finding is unknown and requires further investigation, previous studies have linked trace biogenic amines to a number of psychiatric and neurological disorders (Boulton, 1980; Baker *et al.,* 1993; D'Andrea *et al.,* 2004; D'Andrea *et al.,* 2010). Interstingly the increase in tyramine was not

observed in murine CSF, however 1 of its acidic metabolites, 4-hydroxyphenylacetic acid (HPA) was shown to be increased; this is likely to reflect an increased brain turn over of tyramine. This observation also requires futher investigation. Additional studies should include the analysis of CSF and brain tissue from (1) mice that have been on nitisinone for a longer duration than the current study (*i.e*. 3-6 months) and (2) mice that have had long term nitisinone treatment and are then taken off it (*i.e.* for 1 month). This would establish whether the findings observed in brain tissue in this study were a consequence of the short term exposure to nitisinone (*i.e*. hypertyrosinaemia), and it would show if the changes observed dissappear when nitisinone treament is stopped. Furthermore due to the analytical limitations reported herein regarding the measurement of monoamine neurotransmitters in CSF using liquid chromatography quadrupole time of flight mass spectrometry (LC-QTOF-MS), an alternative more sensitive quantitative approach should be adopted for the analysis of these compounds that are present at low picomolar to nanomolar abundance (*e.g.* liquid chromatography tandem mass spectrometry, (LC-MS/MS)).

A reassuring finding from the work presented herein is that the aromatic amino acids phenylalanine and tryptophan did not change following treament with nitisinone in serum from patients (Chapter 4) and CSF from mice (Chapter 8), supporting that hypertyrosineaemia does not alter uptake or metabolism of these amino acids. This is particularly important as tryptophan is an important substrate for the neurotransmitter serotonin. Together these findings discredit what calculated amino acid ratios (Lieberman, 1999; Ravaglia *et al*., 2004) suggest herein (Chapter 4) with respect to neurotransmitter metabolism (*i.e.* that amino acid availability to the brain may be reduced, with the exception of tyrosine). Whilst this is so treatment with nitisinone clearly has an impact on tryptophan metabolism as the abundance of several urinary and serum tryptophan metabolites were shown to decrease in patients following treatment with nitisinone, with the exception of xanthenuric acid and indole-3-lactate (I-3-L), which increased in urine and serum, respectively.

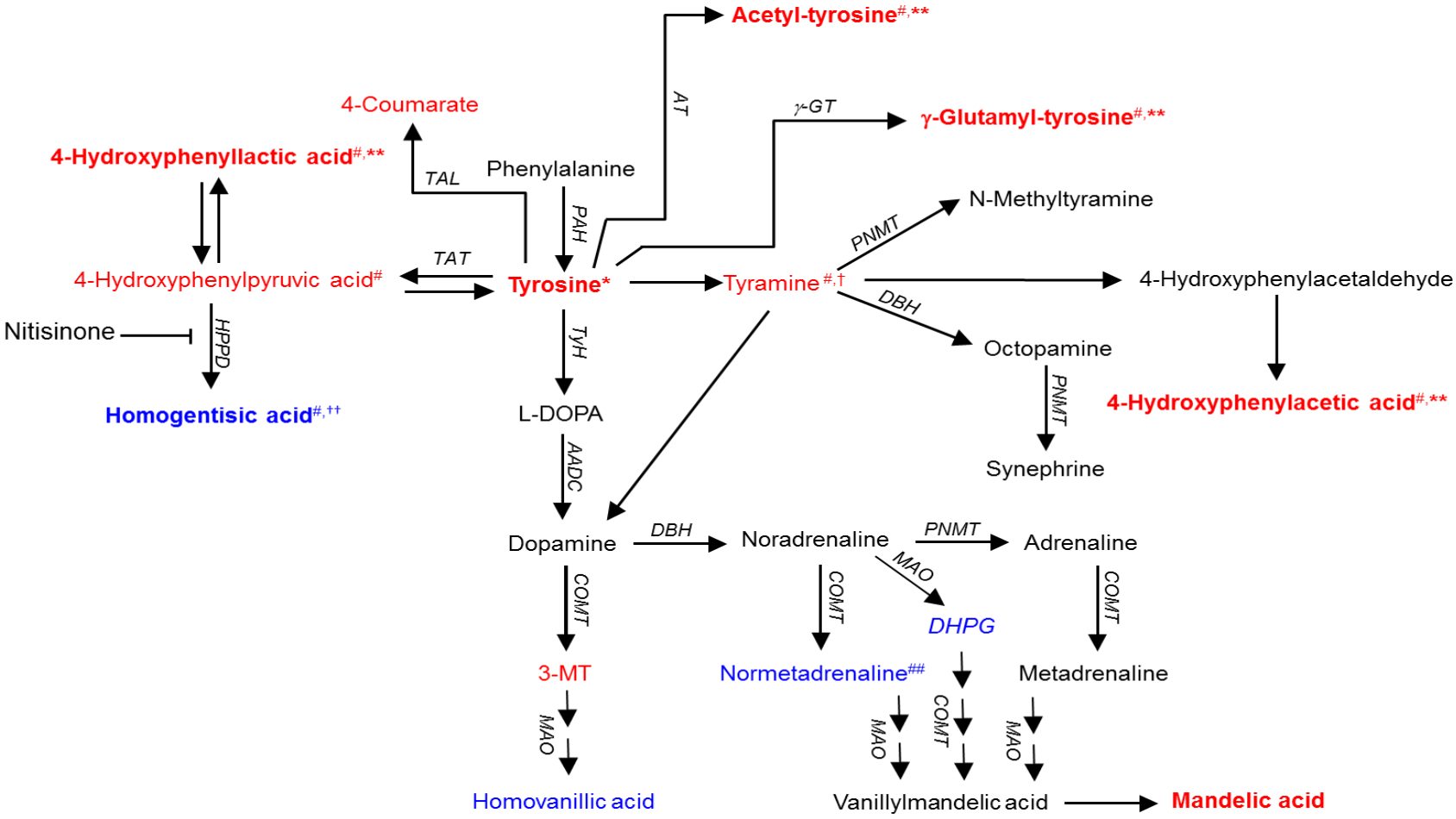
Acetyl-tyrosine#,**
AT
γ-GT
γ-Glutamyl-tyrosine#,**
4-Coumarate
4-Hydroxyphenyllactic acid#,**
TAL
Phenylalanine
PAH
N-Methyltyramine
PNMT
TAT
4-Hydroxyphenylpyruvic acid#
Tyrosine*
Tyramine #,†
4-Hydroxyphenylacetaldehyde
DBH
Nitisinone
HPPD
TyH
Octopamine
Homogentisic acid#,††
L-DOPA
PNMT
4-Hydroxyphenylacetic acid#,**
AADC
Synephrine
Dopamine
DBH
Noradrenaline
PNMT
Adrenaline
MAO
COMT
COMT
COMT
DHPG
3-MT
Normetadrenaline##
Metadrenaline
MAO
COMT
MAO
Homovanillic acid
Vanillylmandelic acid
Mandelic acid

Figure 9.1. Summary of metabolite changes relating to tyrosine and catecholamine neurotransmitter metabolism observed in biological fluids analysed in patients and mice with AKU following treatment with nitisinone. **Red**/**blue** bold text – denotes increase/decrease in abundance in human urine and serum; red/blue non-bold text denotes increase/decrease in abundance in human urine; *blue* italicised text denotes decrease in abundance in mouse urine; * – additionally mouse brain, plasma, urine and CSF; ** – additionally mouse CSF; $^{\#}$ – additionally in mouse urine; † – additionally mouse brain tissue; †† – additionally mouse plasma; $^{\#\#}$ – only in SONIA-1 (Chapter 5); PAH – phenylalanine hydroxylase; TyH – tyrosine hydroxylase; COMT – catechol-O-methyltransferase; MAO – monoamine oxidase; PNMT – phenylethanolamine-N-methyltransferase; DBH – dopamine-β-hydroxylase; AADC – aromatic L-amino acid decarboxylase; TAL – tyrosine ammonia lyase; γ-GT – γ-glutamyltranspeptidase; HPPD – 4-hydroxyphenylpyruvate dioxygenase; AT – acetyl transferase; L-DOPA – L-3,4-dihydroxyphenylalanine; 3-MT – 3-methoxytyramine; DHPG – 3,4-dihydroxyphenylglycol.

The exact mechanism to explain these changes is uncertain and little can be gained from the indiscriminate use of the kynurenine to tryptophan ratio in urine or serum to assess changes in indole-2,3-dioxygenase activity (Badawy *et al.,* 2019). To this end it is not clear whether these findings are (1) an 'off target' effect of nitisinone, (2) a consequence of nitisinone reducing homogentisic acid (HGA) and thus reducing oxidative stress and or (3) a consequence of a metabolite that increases due to the action of nitisinone (*i.e.* keto acids are thought to upregulate tryptophan amino transferase (TAT) activity, Figure 9.2) (Lees *et al.,* 1973; Gertsman *et al.,* 2015b). It is plausible that the latter leads to the majority of changes observed herein, as the increase in I-3-L observed in serum suggests that there is an increased metabolism of tryptophan via the mechanism proposed above. One may hypothesise that the down stream effect of this is that there is less surplus tryptophan available for metabolism via the other major metabolic routes it takes to produce a variety of biologically active compounds (*e.g.* serotonin, tryptamine, kynurenines, and NAD^+), and as a consequence urinary excretion of tryptophan and a number of related metabolites is reduced.

Additionally the increase in I-3-L may also relate to the reduced oxidative stress that has been proposed to occur as a consequence of lowering HGA (Braconi *et al.,* 2010; Braconi *et al.,* 2011; Millucci *et al.,* 2014; Braconi *et al.,* 2015; Davison *et al.,* 2016), thus leading to an increase in the activity of gut microbiota and increased formation of I-3-L. Interestingly a reduction in urinary indoxyl sulphate was observed herein, a metabolite well known to originate from colonic microbiota (Wikoffa *et al.,* 2009), therefore the change observed is not expected. One potential reason for this maybe related to the increased metabolism of tryptophan to I-3-L and as a consequence there maybe less tryptophan available for metabolism to indoxyl sulphate. A reduction in indoxyl sulphate may be percieved as beneficial as it has been implicated as a contributor to the progression of renal and vascular disease. Studies have also suggested that it may also have adverse effects on bones and the central nervous system (Leong *et al.,* 2016).

The significance of the increase in urinary xanthenuric acid in AKU patients following treatment with nitisinone is unknown. This cannot be easily explained by the accumulation of metabolites proximal to xanthenuric acid as they are either lower or unchanged, which would not lead to its increased formation. Moreover this finding cannot be explained by liver disease or vitamin B6 deficiency, which has prevously been associated with increased urinary xanthenuric acid (Rossouw *et al.,* 1978; Ueland *et al.,* 2015).

Another interesting observation was that the abundance of the urinary serotonin metabolite, 5-hydroxyindole acetic acid, decreased following a 8 mg daily dose of nitisinone in the SONIA-1 study (Chapter 5). This was not seen in patients attending the NAC who received long term treatment with nitisinone (2 mg daily dose) (Chapter 6). The reason for this is unknown and the long term metabolic impact of this will be re-assessed in the SONIA-2 clinical trial (ClinicalTrials.gov Identifier: NCT01916382) where patients received a 10 mg daily dose of nitisinone for 4 years.

The rationale above is likely to be an oversimplification as tryptophan metabolism is complex involving the gut and liver, and is distributed into peripheral circulation for transport to the brain, heart, and skeletal muscle, where the free fraction is of biological importance (Cervenka *et al.,* 2017). Moreover an important contributor to tryptophan metabolism is gut microbiota, which are known to generate indole compounds. The increase in indoles observed herein may be beneficial as they act as hydroxyl radical scavengers, neuroprotectants, and selective human aryl hydrocarbon receptor agonists attenuating inflammation (Zelante *et al.,* 2013).

The wider consequence of the changes in tryptophan metabolism are of unknown significance. Of relevance to the CNS, previous studies have shown alterations in tryptophan metabolism in stress-related depression, schizophrenia, Alzheimer's and Parkinson's diseases (Schwarcz *et al.,* 2017). Several kynurenine metabolites have been implicated including quinolinic acid, which is thought to be an important contributor to neuronal excitotoxicity (Stone *et al.,* 1981; Schwarcz *et al.,* 2017);

3-hydroxykynurenine, anthranilic acid and 3-hydroxyanthranilic acid have also been shown to induce neuroinflammation by direct generation of free radical species even under physiological conditions and thus contribute to oxidative stress (Okuda *et al.,* 1998). Herein none of these metabolites were altered; however the decrease in the urinary metabolite 4-hydroxyquinollinic acid may be a reflection of reduced oxidative stress following treatment with nitisinone.

In contrast, kynurenic acid is thought to have neuroprotective properties through its antagonistic action on glutamate receptors (Stone *et al.,* 1981). The decrease in kynurenine observed herein may appear to be detrimental based on this, however one may also see this as beneficial as lower kynurenine may mean there is less substrate for the formation of the toxic metabolites 3-hydroxykynurenine, anthranilic acid, and 3-hydroxyanthranilic acid; which as alluded to earlier can induce oxidative stress.

As changes to tryptophan metabolism have been observed peripherally one cannot relate changes directly to the CNS, but should be mindful that dysregulation of kynurenine metabolism in peripheral tissues can also have a strong effect on the CNS, resulting in complications such as anxiety and depression (Cervenka *et al.,* 2017).

It has already been highlighted that some of the changes observed in the biological matrix studies may relate to changes in the redox state of the body that occur as a consequence of the reduced HGA observed following treatment with nitisinone (Figure 9.3). Additional changes to the abundance of metabolites reported herein were observed in purine metabolism (Figure 9.3 (A)), glutathione and intermediary metabolism (Figure 9.3 (B)) and the gut microbiome (Figure 9.3 (C)).

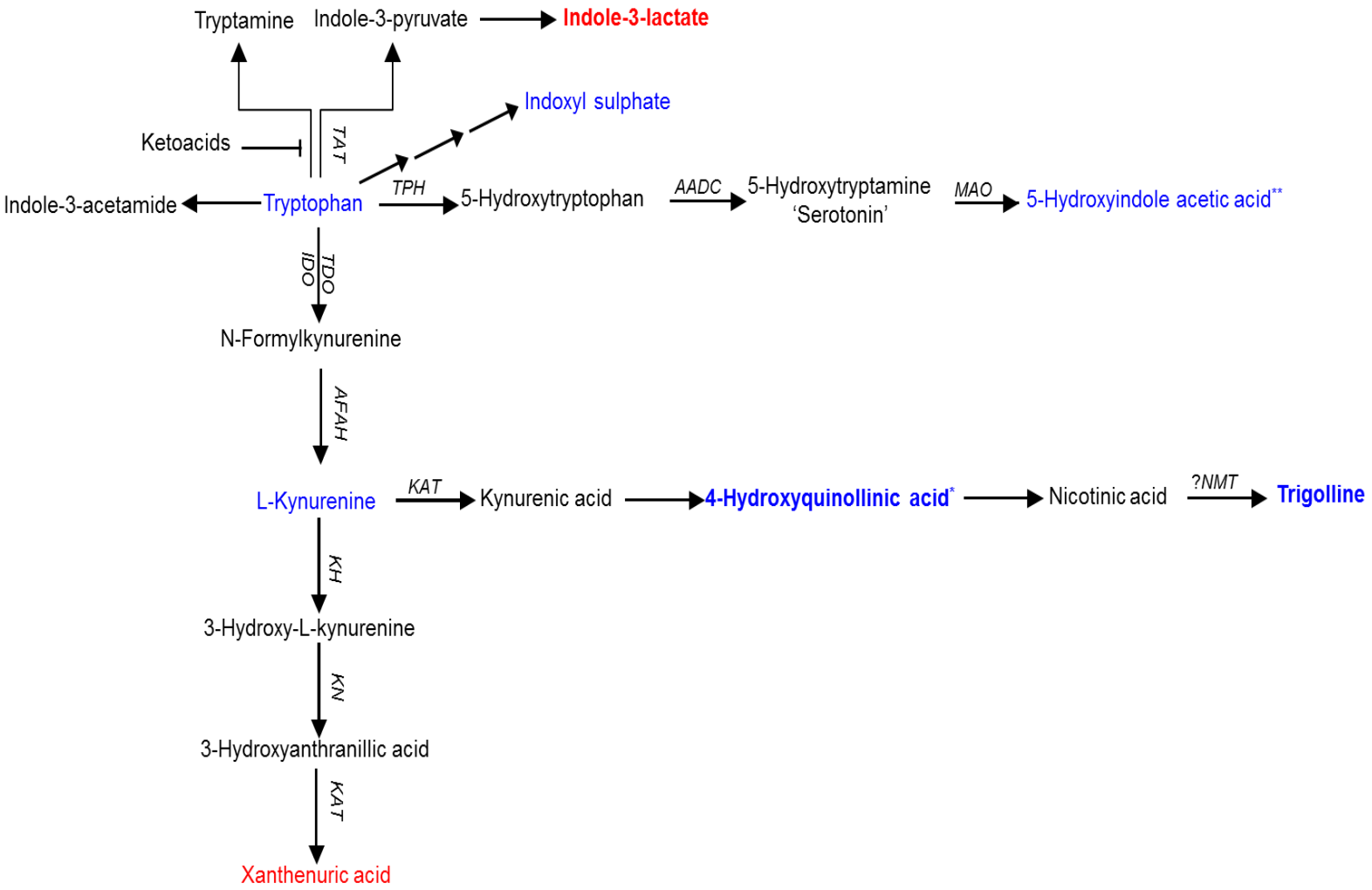
Tryptamine
Indole-3-pyruvate
Indole-3-lactate
Indoxyl sulphate
Ketoacids
TAT
Indole-3-acetamide
Tryptophan
TPH
5-Hydroxytryptophan
AADC
5-Hydroxytryptamine
'Serotonin'
MAO
5-Hydroxyindole acetic acid**
IDO
TDO
N-Formylkynurenine
AFAH
L-Kynurenine
KAT
Kynurenic acid
4-Hydroxyquinollinic acid*
Nicotinic acid
?NMT
Trigolline
KH
3-Hydroxy-L-kynurenine
KN
3-Hydroxyanthranillic acid
KAT
Xanthenuric acid

Figure 9.2. Summary of metabolite changes relating to tryptophan and serotonin neurotransmitter metabolism observed in biological fluids analysed in patients and mice with AKU following treatment with nitisinone. Red/blue – denotes increase/decrease in abundance; **bold** text – refers to change in human serum; non-bold refers to human urine; *- change also observed in mouse urine; **– only observed in SONIA-1 study at a dose of 8 mg daily nitisinone (Chapter 5); AADC – aromatic L-amino acid decarboxylase; AFAH – aryl-formylamine amidohydrolase; ALDH – aldehyde dehydrogenase; KAT – kynurenine aminotransferase; KH – kynurenine hydroxylase; MAO – monoamine oxidase; TDO – tryptophan dioxygenase; IDO – indole dioxygenase; TPH – tryptophan hydroxylase; KN – kynureninase; NMT – N-methyltransferase; TAT – tryptophan amino transferase (proposed enzyme keto acids inhibit).

A decrease in the abundance of several metabolites relating to purine metabolism were observed, principally in urine with the exception of caffeate which increased. Purines are derived from both endogenous metabolism and exogenous dietary intake, with the uric acid being the final oxidation production of metabolism. Increases in circulating uric acid concentrations are widely reported in gout, and have been correlated with cardiovascular disease, hypertension, and renal disease (Chen *et al.,* 2016). It has been postulated that hyperuricemia contributes to the progression of cardiovascular disease through oxidative stress, systemic inflammation, and endothelial dysfunction (Borghi *et al.,* 2016). Thus one may hypothesise that a reduction in metabolites may reduce oxidative stress.

Patients included in this study were not on uric acid-lowering medication, however they were on a protein-restricted diet which may have contributed to the decrease in metabolite abundance observed. A more plausible explanation relates to changes in the oxidative state of the body. Urinary allantoin, which was lower following nitisinone treatement, has previously been suggested as a marker of oxidative stress (Marrocco *et al.,* 2017); one may postulate that the decreases observed herein are a reflection of reduced oxidative stress in the face of lower HGA concentrations (Braconi *et al.,* 2010; Braconi *et al.,* 2011; Millucci *et al.,* 2014; Braconi *et al.,* 2015; Davison *et al.,* 2016). The decrease may also be a consequence of the increase in caffeate observed, which is known to inhibit the activity of xanthine oxidase (EC 1.17.3.2), the enzyme responsible for the metabolism of hypoxanthine and xanthine (Chang *et al.,* 1994). The reason for seeing this increase in caffeate is unknown, but one may postulate that it may be an ‘off target’ effect of nitisinone. Nevertheless the increase in caffeate may be benefical to patients with AKU as it has antioxidant properties (Chen *et al.,* 1997).

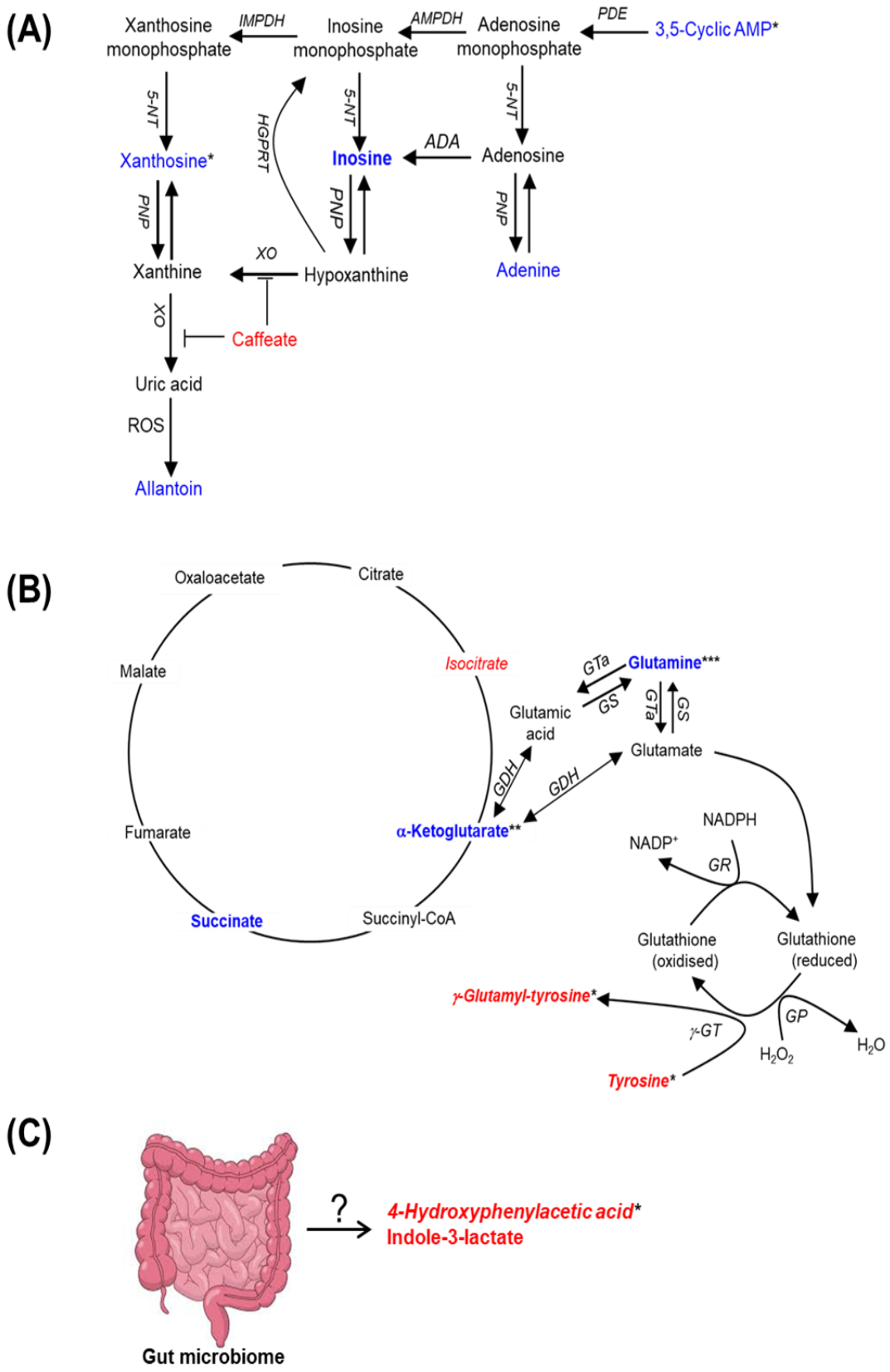

Figure 9.3. Summary of metabolite changes proposed to relate to oxidative stress observed in biological fluids analysed from patients and mice with AKU following treatment with nitisinone. (A) Purine

metabolism; (B) Glutathione and intermediary metabolism and (C) Gut microbiome. Red/blue text – indicates increase/decrease in abundance; **bold** text – change observed in serum from AKU patients; ***bold italicised*** – change observed in serum and urine from AKU patients; *non bold italicised* – change observed in AKU mouse urine; *– change also observed in AKU mouse urine; ** – increase observed in mouse urine; *** – decrease observed in serum using HPLC (Chapter 4) not LC-QTOF-MS (Chapters 3). XO – xanthine oxidase; IMPDH – inosine monophosphate dehydrogenase; AMPDH – adenosine monophosphate dehydrogenase; PDE – phosphodiesterase; 5-NT – 5-nucleotidase; PNP- purine nucleotide phosphorylase; HGPRT – hypoxanthine guanine phosphoribosyltransferase; ROS – reactive oxygen species; GTa – glutaminase; GS – glutamine synthetase; GDH – glutamate dehydrogenase; GR – glutathione reductase; GP – glutathione peroxidase; γ-GT – γ-glutamyltranspeptidase.

The increase in γ-glutamyl-tyrosine in urine, serum and CSF from AKU patients and mice suggests that glutathione metabolism and the redox state of the cell (Griffith *et al.,* 1979; Zhang *et al.,* 2009) are altered following treatment with nitisinone (Figure 9.3 (B)). One may hypothesise that the reduced burden on the glutathione cycle improves glutathione availability, thus enabling the transfer of the glutamyl moiety from glutathione to tyrosine (Griffith *et al.,* 1979; Zhang *et al.,* 2009). Changes in the abundance of some citric acid metabolites were also observed in serum from AKU patients, and urine from AKU mice following nitisinone treatment, suggesting that intermediary energy metabolism is likely to be an 'off target' effect of treatment with nitisinone.

Previously Hallan *et al*. (2017) reported on the clinical utility of measuring citric acid metabolites in a cohort of non-diabetic patients with chronic kidney disease, suggesting that their measurement in serum and urine are a direct reflection of citric acid cycle activity. Herein serum α-ketoglutaric, glutamine and succinic acid decreased in AKU patients (Figure 9.3 (B)), suggesting that

metabolites may be diverted to facilitate glutathione production and recycling. Interestingly a contradictory picture was observed in mouse urine, as α-ketoglutaric acid and isocitrate were increased. This relationship requires further investigation, but one may postulate serum is a better reflection of internal homeostasis, moreover the changes in mouse urine were observed in a random spot sample and not a complete 24 h sample, like in AKU patients. Further investigations are required to better understand this relationship as it is clear that citric acid metabolites have a key role to play in health and disease (Martínez-Reyes *et al*., 2020).

The increase in HPA in serum and urine following nitisinone treatment is a curious finding in humans as it has not been reported to date. Unlike in the CSF, it is likely to originate from gut microbiota (Liu *et al.,* 2016), and again one may hypothesise that the increase observed is a consequence of less oxidative stress following nitisinone, which increased microbiotic metabolism.

The changes in 4-hydroxybenzaldehyde and benzaldehyde are highly novel findings and it is possible they are related to benzoquinones; one may postulate they are intermediates in the formation of ochronotic pigment observed in AKU. Interestingly recent reports have suggested that there are therapeutic benefits from benzaldehyde compounds (Lee *et al.,* 2010; Moon *et al.,* 2012; Kong *et al*., 2014; Lee *et al.,* 2014).

A number of other changes in the abundance of urinary and serum metabolites were observed in the studies presented here. Evaluation of their significance presents a significant challenge as there is no obvious or theoretical relation to the action of nitisinone or AKU as a disease entity. To this end one may postulate that many of these are ‘off target’ effects of nitisinone. These include glycocholate, homoserine, mevalolactone and allothreonine in serum from AKU patients; 5-valeroclactone, creatine, maleimide, N-acetylglycine in mouse urine; ethylmalonic acid, 2-hydroxybutyric acid, L-threonine, methyl vanillate in urine from AKU patients and 2-hydroxy-4-(methylthio)butyric acid which was observed in urine from mice and AKU patients.

There are some confounding factors to consider in the body of data presented in this thesis. Firstly in relation to the impact of hypertyrosinaemia on neurotransmitter metabolism; analysis of serum and urine samples following treatment with nitisinone cannot give a direct answer to this question. It can however provide invaluable indirect evidence, and reassuringly the findings of these analyses in this study are corroborated by the observations from the direct analysis of CSF and brain tissue from a well characterised mouse model of AKU. The findings of the latter are however incomplete owing to the limited analytical sensitivity of the LC-QTOF-MS method used for the analysis of CSF. Future studies should consider the use of quantitative LC-MS/MS methodology as it is a more sensitive technique for the measurement of neurotransmitter compounds present at low picomolar to nanomolar concentrations. This will however be met with its own challenges as method validation and sample analysis will be limited by the volume of CSF that can be collected from mice. It is also unlikely that ethical permission will be sought to collect CSF from patients with AKU as they do not demonstrate clinical features of depression or altered mood following treatment with nitisinone. Also as these patients have complex musculoskeletal comorbidities sample collection is high risk and technically challenging.

Secondly, whilst this is the most comprehensive evaluation of the metabolome reported in AKU following treatment with nitisinone additional work is required to provide a better understanding of the ‘off target’ effects of this drug. It is clear that its action in blocking the activity of HPPD has wider metabolic consequences beyond hypertyrosinaemia, some of which are surprising. Moreover the work presented herein, with the exception of urine samples from the SONIA-1 study, has looked at the impact of treatment with nitisinone in patients that have attended the NAC without simultaneously studying a group of AKU patients that did not receive treatment (*i.e.* no treatment control group). To this end one may speculate that some of the changes observed may relate to the evolution of AKU as a disease and not as a consequence of nitisinone treatment.

Additionally whilst metabolomics data were acquired using non-targeted LC-QTOF-MS methods, data analysis was performed using a targeted feature extraction method. Though this method was comprehensive covering a broad range of metabolites it precluded from the identification of novel metabolites that may have been observed following treatment. Future studies should include the non-targeted interrogation of data.

Looking to the future it is hoped that the novel findings presented herein, as well as its shortcomings will be addressed in the SONIA-2 clinical trial (ClinicalTrials.gov Identifier: NCT01916382), which was completed recently. This trial includes the largest cohort to date of AKU patients from Europe (excluding the United Kingdom), which have been randomised to receive no treatment or a 10 mg daily dose of nitisinone over a 4 year period. It is hoped that this invaluable trial and the work presented herein will improve the lives of patients with AKU, and that the analytical platform created through AKU for clinical metabolomics in Liverpool will be applied to study the metabolic basis of other diseases. It seems very fitting to finish by quoting Archibald Garrod ('The Scientific Spirit in Medicine: Inaugural Sessional Address to the Abernethian Society', St. Bartholomew's Hospital Journal, 1912 20:19):

“Nevertheless, scientific method is not the same as the scientific spirit. The scientific spirit does not rest content with applying that which is already known, but is a restless spirit, ever pressing forward towards the regions of the unknown, and endeavouring to lay under contribution for the special purpose in hand the knowledge acquired in all portions of the wide field of exact science. Lastly, it acts as a check, as well as a stimulus, sifting the value of the evidence, and rejecting that which is worthless, and restraining too eager flights of the imagination and too hasty conclusions.”

Chapter 10

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Gene database: https://www.ncbi.nlm.nih.gov/gene

HGD mutation database: http://hgddatabase.cvtisr.sk

Human Metabolome Database: http://www.hmdb.ca

KOMP repository – knockout mouse project: www.komp.org

MetaboAnalyst: https://www.metaboanalyst.ca/

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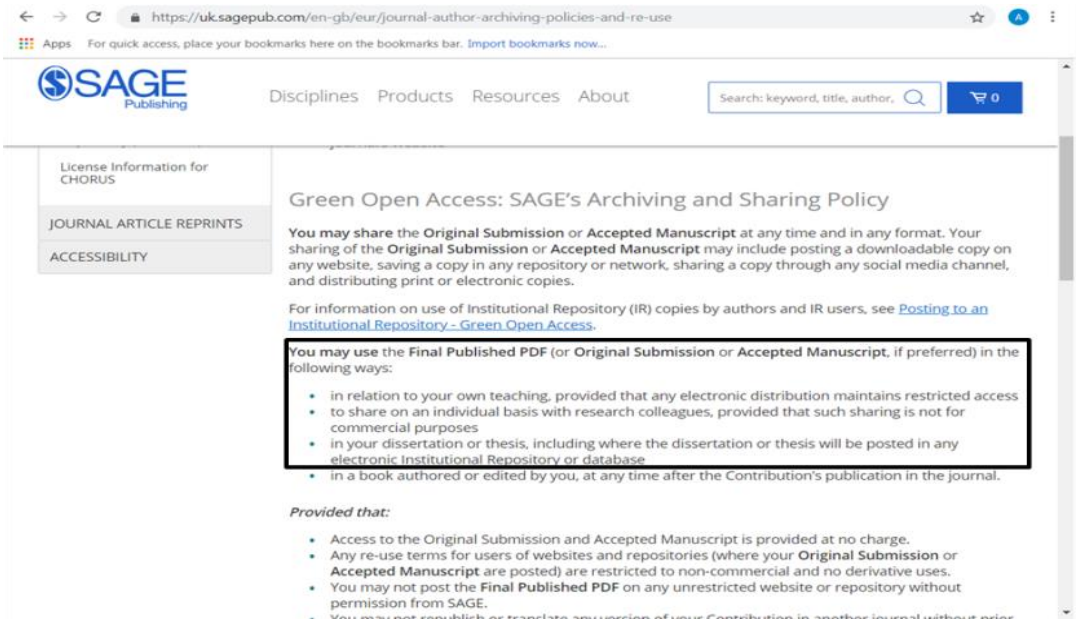
Article: Alkaptonuria - many questions answered, further challenges beckon. Ann Clin Biochem 2019;doi:10.1177/0004563219879957.



i.ii. Journal of Inherited Metabolic Disease

Article: Acute fatal metabolic complications in alkaptonuria. J Inherit Metab Dis 2016;39(2):203-21.

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(ii) Chapter 2

ii.i Clinical Chemistry

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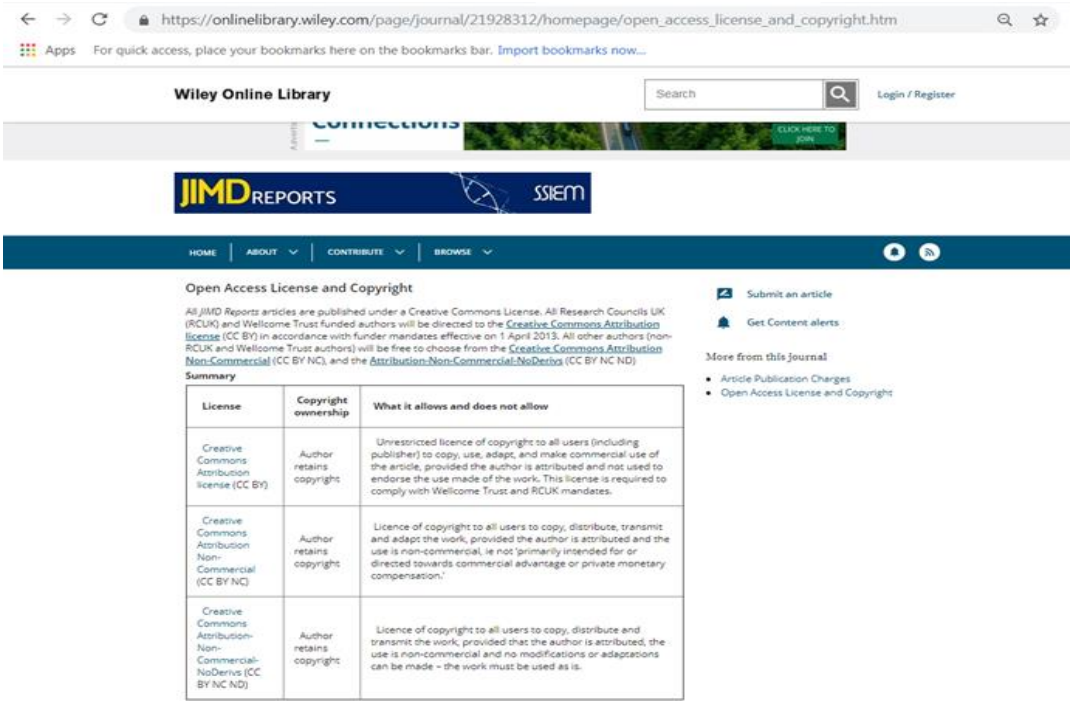
(iii) Chapter 3

iii.i Journal of Inherited Metabolic Disease Reports

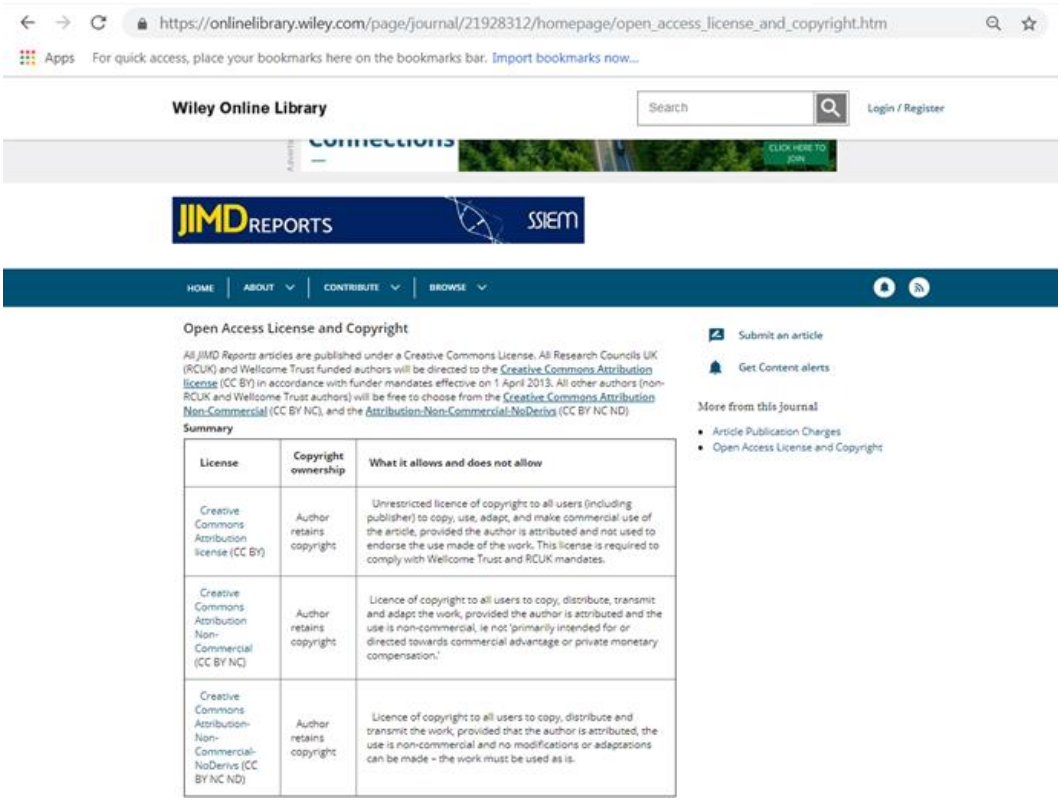
Article: Evaluation of the serum metabolome of patients with alkaptonuria before and after 2 years of treatment with nitisinone using LC-QTOF-MS. JIMD Rep 2019;48:67–74.



(iv) Chapters 4

iv.i Journal of Inherited Metabolic Disease Reports

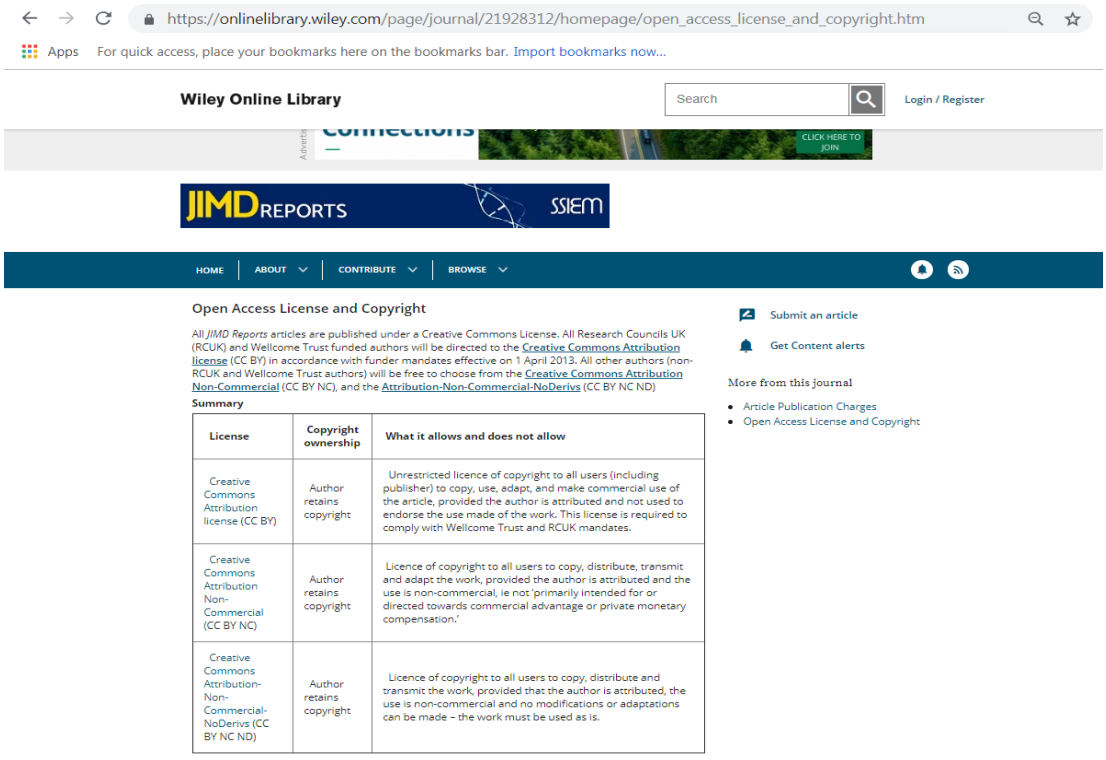
Article: Serum amino acid profiling in patients with alkaptonuria before and after treatment with nitisinone. JIMD Rep 2018;41:109-117.



(v) Chapters 5

v.i Journal of Inherited Metabolic Disease Reports

Article: Assessment of the effect of once daily nitisinone therapy on 24 hour urinary metadrenalines and 5-hydroxyindole acetic acid excretion in patients with alkaptonuria after 4 weeks of treatment. JIMD Rep 2018;41:1-10.



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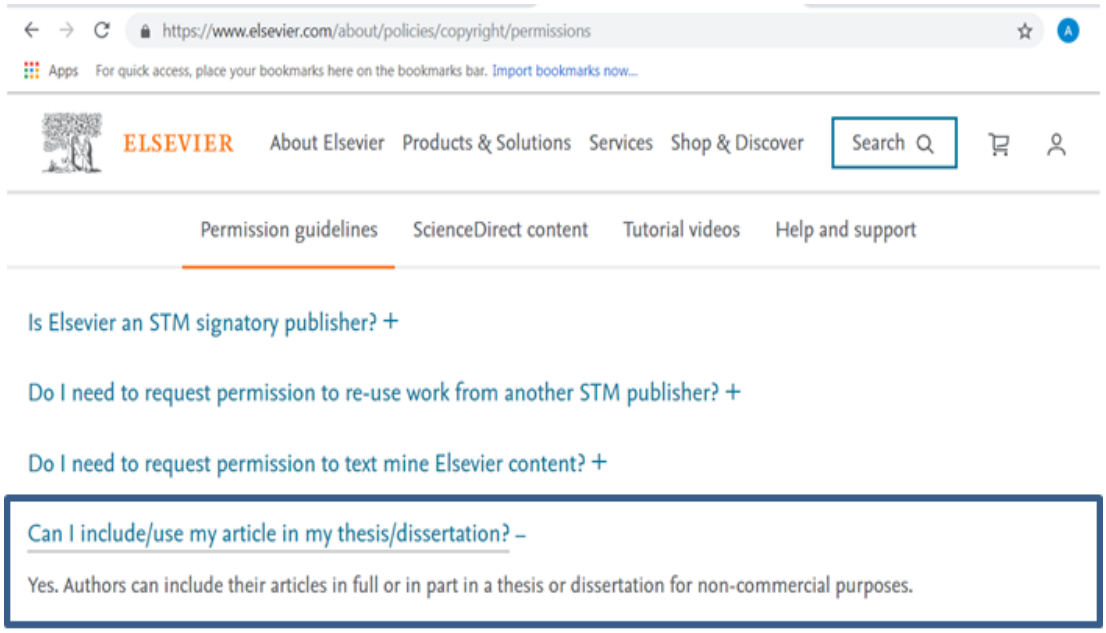
(vi) Chapter 6

vi.i Molecular Genetics and Metabolism

Article: Clinical and biochemical assessment of depressive symptoms in patients with alkaptonuria before and after 2 years of treatment with nitisinone. Mol Genet Metab 2018;125(1-2):135-143.



(vii) Chapter 7

vii.i Metabolomics

Article: Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging. Metabolomics 2019;15(68):1-10.

https://link.springer.com/article/10.1007/s11306-019-1531-4

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(viii) Chapter 2 and 3

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02/01/2020

To whomever it may concern:

Permission to include jointly authored publication into PhD thesis: 'Biochemical investigations into the impact of nitisinone treatment on the metabolome and assessment of whether nitisinone induced hypertyrosinaemia alters neurotransmitter metabolism'

With this letter I confirm that I give my permission to Andrew S. Davison (affiliated with University of Liverpool and the Royal Liverpool University Hospitals NHS Foundation Trust) to include our jointly authored manuscripts into his PhD thesis for submission in January 2020. The related publications are:

(1) A comprehensive LC-QTOF-MS metabolic phenotyping strategy: application to alkaptonuria. Clinical Chemistry 2019;65(4):530-539.

(2) Evaluation of the serum metabolome of patients with alkaptonuria before and after 2 years of treatment with nitisinone using LC-QTOF-MS. Journal of Inherited Metabolic Disease Reports 2019;48:67-74.

Yours sincerely,

Brendan P Norman

(ix) Chapter 7

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To whomever it may concern:

Permission to include jointly authored publication into PhD thesis

With this letter I confirm that I give my permission to Andrew S. Davison (affiliated with the Royal Liverpool University Hospitals Trust and the University of Liverpool) to include our jointly authored manuscript "Assessing the effect of nitisinone induced hypertyrosinaemia on monoamine neurotransmitters in brain tissue from a murine model of alkaptonuria using mass spectrometry imaging" published in the journal Metabolomics (DOI: 10.1007/s11306-019-1531-4) into his PhD thesis titled "Biochemical investigations into the impact of nitisinone treatment on the metabolome and assessment of whether nitisinone induced hypertyrosinaemia alters neurotransmitter metabolism" for submission in January 2020.

Kind regards,

Nicole Strittmatter, Cambridge 31.12.2019